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INHERITANCE OF CAROTENOID DIFFERENCES IN THE TOMATO HYBRID YELLOW × TANGERINE

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YELLOW varieties of tomatoes have been known since the crop was first introduced into Europe shortly after the discovery of America, and were probably to be found in Mexico before the conquest (JENKINS 1949). HALSTED et al. (1905) reported on the red × yellow hybrid, establishing the fact that the color difference was due to segregation at a single locus, a conclusion that has been confirmed repeatedly by subsequent workers. Presumably, the yellow arose as a mutant from the red, and probably the mutation occurred on several occasions. RICK (private communication) has recently found a yellow mutant in the variety Pearson.

The tangerine or orange tomato has a more obscure history. MACARTHUR (1934) lists, red fruit flesh (*T*) *v.* tangerine (*t*) in his table to tomato character contrasts (p. 124). He also states, " The monofactorial inheritance of the characters [listed in the table] has been definitely proven with adequate numbers and suitable tests." He refers to an earlier paper (1931) which contains data on the segregation of most characters listed in his 1934 paper. However, we found no reference to the tangerine tomato in his 1931 paper.

Apparently, MACARTHUR (1934) was the first to study segregation in the yellow × tangerine hybrid, but his report on this hybrid is incidental to a presentation of the linkage relations between various loci, and consists of three brief references. First, " The factor interactions of the two types of dwarfness lead to 12 : 3 : 1 ratios in F_2; those between the two forms of green stem and the two flesh colors produce 9 : 3 : 4 ratios " (p. 125). Secondly, in table III (p. 128) the crossover value between the yellow and tangerine loci is given as 52.1 ± 2.5, which indicates that he made the yellow-tangerine hybrid. Finally, in tables IX and X (p. 130) he gives additional crossover data from which he concludes that the tangerine locus is in linkage group VII with the two loci, uniform fruit and hairy stem, both of which show independent assortment with yellow in linkage group II.

FLEMING and MYERS (1938) through correspondence with MACARTHUR, were able to establish the latter's F_2 ratio as 9 red : 3 yellow : 4 orange (3 orange and 1 light orange). However, in their own data on the yellow-tangerine hybrid they obtained results which they interpreted in a somewhat more complicated manner. In backcrosses between the F_1 and the parents of the yellow × tangerine hybrid, they obtained the expected 1 : 1 ratios. In F_2, on the other hand, they report 459 red : 134 yellow : 179 orange. The significant excess of reds and deficiencies of yellows and oranges are not in accord with MACARTHUR's hy-

pothesis. Furthermore, the latter hypothesis, as the authors express it, " [does not] account for the presence of red-orange and orange-yellow combinations in the same fruit." Consequently, FLEMING and MYERS proposed a much more elaborate hypothesis to account for their backcross and F_2 segregations. They postulated in addition to the two basic color genes, tangerine and yellow, two modifying factors and two specific inhibitors.

In a chemical analysis by means of the chromatographic technique, LEROSEN et al. (1941) compared red (*RR*) and yellow (*rr*) tomatoes. They concluded that the *R* gene is responsible for an increase in red and yellow plastid pigments, chiefly lycopene and, to a much lesser extent, carotenes and xanthophylls. ZECHMEISTER et al. (1941) in their study of the tangerine tomato (*RRtt*) found a series of lycopene isomers, the most prominent of which was a new pigment, prolycopene.

MACKINNEY and JENKINS (1949) in reporting on the carotenoids of several strains of red, yellow and tangerine tomatoes found that some strains of yellow tomatoes did produce small amounts of lycopene, and also pointed out that while prolycopene determines the color of the tangerine tomato, its absorption spectrum is dominated by pigments with maxima between 430 and 400 mμ, which is accounted for by two pigments, all-trans zeta-carotene and a carotenoid which they designated as poly-cis psi-carotene. This latter pigment (which bears some relationship to the unidentified carotene I of PORTER and ZSCHEILE (1946) and to zone 16 of LEROSEN and ZECHMEISTER (1942)), and prolycopene are unique to the tangerine tomato. Zeta-carotene as a major component is also restricted to the tangerine.

A study of the yellow × tangerine hybrid was begun in 1947, and a partial account of the yellow × tangerine hybrid, stressing the biochemical aspects, was given by MACKINNEY and JENKINS (1952). The present paper will deal in greater detail with the genetic aspects of the study, but will also include sufficient biochemical data to discuss the role of the yellow and tangerine loci in carotenogenesis.

MATERIALS AND METHODS

The female parent (*rrTT*) of the original hybrid was an inbred tester line developed by E. W. LINDSTROM, which in addition to being homozygous at the yellow locus, contained the following recessive genes in the homozygous condition: $dwarf_1$, peach, oval, compound inflorescence. The male parent (*RRtt*) was the commercial variety Jubilee, which in addition to being tangerine had uniform and colorless fruit-skin. The yellow parent had small, two-loculed fruits ranging from 15 to 50 grams in weight. The tangerine, on the other hand, had much larger multilocular fruits ranging up to 250 grams. In order to avoid contamination, all of the hybridizations were carried out in an insect-free greenhouse. In fact, most of the segregating generations were grown in six- or eight-inch pots in the same greenhouse.

Details of the methods used in determining the carotenoid content of the fruits are given by MACKINNEY and JENKINS (1949, 1952)

EXPERIMENTAL RESULTS

As was to be expected from the fact that MACARTHUR had placed the yellow and tangerine loci on different chromosomes, the fruit color in the F_1 generation of the yellow-tangerine hybrid was red. This result was also in conformity with the data of FLEMING and MYERS. Furthermore, these F_1 fruits could not be distinguished from those of typical red varieties either from their phenotypic appearance or on the basis of their carotenoid content.

The classification of the F_2 phenotypes, on the other hand, presented several difficulties, and it was not until data from chemical analysis were obtained that a satisfactory classification of the F_2 could be made. It is clear from the last line of table 1 that the F_2 segregated in the ratio of 9 red : 3 yellow : 4 orange.

TABLE 1

Segregation of fruit colors in the F_2 of the Yellow × Tangerine hybrid.

Culture		Red	Yellow	Orange	Total	Tangerine	Yellow-tangerine
		9	3	4		3	1
1	Obs.	14	1	5	20	5	0
	Calc.	11.25	3.75	5.00	20	3.75	1.25
2	Obs.	7	5	4	16	4	0
	Calc.	9.00	3.00	4.00	16	3.00	1.00
3	Obs.	11	1	8	20	4	4*
	Calc.	11.25	3.75	5.00	20	3.75	1.25
4	Obs.	10	2	2	14	1	1
	Calc.	7.88-	2.62	3.50	14	2.62	0.88
5	Obs.	8	8*	5	21	4	1
	Calc.	11.81	3.94	5.25	21	3.94	1.31
Total	Obs.	50	17	24	91	18	6
	Calc.	51.19	17.06	22.75	91	17.06	5.69

*Large deviations from expectation.

Furthermore, the orange class could be further subdivided into two classes: a tangerine class, which had the same carotenoid content as the tangerine parent and a yellow-tangerine class, which had a distinctly different carotenoid content. Thus we were able to confirm MACARTHUR'S ratio of 9 red : 3 yellow : 3 tangerine : 1 light tangerine (yellow-tangerine).

Since it was planned to analyze the carotenoid content of the segregating generations on an individual plant basis, the F_2 were grown in small cultures of about twenty plants each. The first difficulty that arose was that the yellow-tangerine (*rrtt*) did not appear until the third of these cultures had been grown (table 1). This late appearance as well as the sporadic occurrence of other non-red segregates in the various cultures has been attributed to chance. The totals fit MACARTHUR'S ratio and, in addition, there is no evidence of heterogeneity between cultures ($\chi^2 = 14.99$ with 8 d.f., $P = 0.10$–0.05). However, in testing

the homogeneity of the cultures on the basis of a 9:3:3:1 ratio, there is some evidence against the hypothesis that all cultures come from the same population ($\chi^2 = 22.49$ with 12 d.f., P = 0.05–0.02). But, the deviations are not large, with two exceptions which are indicated by an asterisk in table 1.

The second and most serious difficulty that arose in classifying the F_2 generation was due to environmental variability. Because the cultures were not all grown simultaneously but scattered throughout the year, there was considerable variability in the color of the tangerine fruits and more particularly in the yellow-tangerines. Plants grown during the winter season had a somewhat higher carotenoid content, a slightly different distribution of pigments and a darker color than those grown during the summer. In fact, cuttings from the same plant grown at different seasons often had quite distinct fruit colors. Some of the yellow-tangerines were so light that they could easily be mistaken for yellows and others were so dark that without chemical analysis they could easily be considered as tangerines. Even some of the tangerines were sufficiently dark to be mistaken for reds.

Finally, segregation at other loci, principally the colorless-skin locus, was responsible for variability in genotype both within and between cultures. As pointed out by SMITH and SMITH (1931) high temperatures and high light intensity either inhibit the production or hasten the destruction of carotenoid pigments. Plants homozygous for the colorless skin gene had greater variation in fruit color. There was often less intense pigmentation immediately under the skin in various parts of the fruit, which often resulted in a mottled appearance. Furthermore, in the F_2 generation there was a great range of fruit size which also led to considerable difficulty in classification because, as a rule, small fruits are more intensely pigmented than large fruits.

Due to environmental variability and to segregation at other loci, the phenotypic appearance was not always a reliable indicator of the genotype. Consequently, in order to confirm the F_2 classification, individual F_2 plants were selfed for the purpose of studying segregation in their progeny. In addition, several tangerine and yellow-tangerine plants were crossed to the yellow parent to determine whether or not they contained the recessive allele *r*. Similarly, a yellow was crossed with the tangerine parent in order to test for the presence of the *t* allele (table 3).

In all cases F_3 cultures from individual F_2 plants segregated as expected on the basis of their F_2 classification (table 2). The progenies from red-fruited F_2 plants were of different types, and together they segregated all four phenotypes: red, yellow, tangerine and yellow-tangerine. The single yellow F_2 tested gave only yellow F_3 progeny. Tangerines as a group segregated both tangerines and yellow-tangerines. It is possible that the two cultures which had only tangerine progeny were of the parental genotype (*RRtt*). However, the numbers are too small to draw definite conclusions. Finally, the yellow-tangerines had only yellow-tangerine progeny.

Similarly, the test crosses (table 3) behaved as predicted on the basis of their F_2 classification. The tangerines were of two types, *RRtt*, which only

gave red progeny on crossing with the yellow parent, and *Rrtt*, which gave half red and half yellow when crossed with the yellow parent. The yellow-tangerines *rrtt* gave only yellow progeny when crossed with the yellow parent. The only yellow F_2 tested by crossing with the tangerine parent, gave all red progeny indicating that it had the genotype *rrTT*.

In conclusion, all of the data conform to MACARTHUR's 9 red : 3 yellow : 4 tangerine (orange). If the yellow-tangerine, which may at times be as highly colored as the tangerine, is considered to be identical with MACARTHUR's light tangerine (light orange), there is complete agreement with the latter's

TABLE 2

Segregation of fruit colors in F_3 progenies from F_2 selfed plants.

F_2 parent	F_3 progenies							
Phenotype	Red		Yellow		Orange			Total
	Anal.	Not anal.	Anal.	Not anal.	Analyzed		Not anal.	
					Tang.	Y-tang.		
Red	2	2	1	3				8
"		1						1
"		6		1	1		2	10
"		5		2	1	1		9
"		6		1	1	1		9
"		6		1			1	8
Yellow			2	1				3
Tangerine					1	2	4	7
"					2	1	4	7
"					2		2	4
"					2		2	4
"					1	1	5	7
"					2	1	5	8
"					1	3	1	5
Yellow-Tang.						9	1	10
"						6	1	7
"						3	6	9
"						6	3	9
						7		7

9:3:3:1 ratio. The failure of FLEMING and MYERS (1938) to confirm MACARTHUR's results in their F_2 generation could very well be due to a combination of variability of the environment and to segregation at genes at other loci, especially since they obtained the expected 1 : 1 ratios in their backcross generations. In any case, their elaborate explanation does not apply to the present data.

The distribution of the analyzed carotenoids in the various phenotypes has been reported already (MACKINNEY and JENKINS 1952). As far as our observations go there is complete dominance of the *T* and *R* alleles; in other words, we could detect no difference between the various red genotypes and similarly

for the two genotypes within the yellow and tangerine phenotypes. The variability within phenotypes is due to other causes, such as environment, genetic background and experimental error. The most surprising result of the analysis was the high total carotenoid content of the yellow-tangerine. That is, the double recessive has a higher content than the yellow. This indicates that the low production of total carotenoid associated with *rr* does not affect all carotenoids equally. All-trans lycopene, in particular, is strikingly reduced in amount by the substitution of *rr* for *RR* or *Rr*.

Red. The predominant pigment of red tomatoes, and this applies to red varieties generally as well as to the red segregates in this hybrid, is all-trans lycopene and lesser amounts of beta-carotene and phytofluene respectively, as well as trace amounts of other carotenoids.

TABLE 3

Segregation of fruit colors in test crosses.

F_2 parent		Test progenies		
Phenotype	Genotype	Red	Yellow	Total
Tangerine[1]	*RRtt*	10	....	10
"	"	6	....	6
"	"	10	....	10
"	*Rrtt*	2	4	6
"	"	5	5	10
Yellow-Tang.[1]	*rrtt*	....	10	10
"	"	....	10	10
Yellow[2]	*rrTT*	9	....	9

[1]The tangerine and yellow-tangerine F_2 plants were crossed with the yellow parent (*rrTT*).

[2]The yellow F_2 plant was crossed with the tangerine parent (*RRtt*).

Yellow. The most striking feature of the yellow tomato is its very low total carotenoid content; and the reduction is most pronounced in the case of all-trans lycopene.

Tangerine. The characteristic pigment in the tangerine tomato is prolycopene, which gives the peculiar orange color to this phenotype. In addition to prolycopene there are varying amounts of mono-cis lycopenes. Since these have a red color, they are largely responsible for the variability in color of the tangerine tomato, which in extreme cases makes it almost indistinguishable from a red tomato. In addition to differences in the lycopene set (the absence of all-trans lycopene and the presence of prolycopene and mono-cis isomers), the tangerine differs from the red most strikingly in its content of psi-carotene and zeta-carotene. The content of beta-carotene and phytofluene does not differ markedly between the two. Thus *tt* does not alter the total content, but has a marked effect on several different carotenoids.

Yellow-tangerine. The double recessive is essentially a tangerine, since, like the latter, it contains prolycopene and mono-cis isomers. However, unlike the

tangerine it contains no psi-carotene and only a trace of zeta-carotene. The reduction in carotenoid production associated with *rr* is obvious, although the total reduction is not so great as in the case of the yellow. Nevertheless the latter has a higher content of beta-carotene, so that *rr* interferes with the production of all carotenoids, but the interference is selective in that some carotenoids are affected more than others.

DISCUSSION

The obvious first approach in determining the role of mutant genes is to look for specific blocks in the biosynthetic chain, which are usually detected by the accumulation of an intermediate. ZECHMEISTER et al. (1941) suggested that prolycopene was such an intermediate and further suggested that the *R* allele is involved in the synthesis of prolycopene and that the *T* allele is responsible for the conversion of prolycopene to lycopene in red tomatoes. However, the tangerine tomato has large amounts of mono-cis lycopene and poly-cis psi-carotene (which we have not been able to detect in the red tomato), as well as considerably larger amounts of zeta-carotene. Consequently, there seems to be no reason to single out prolycopene as the specific precusor of lycopene. WENT et al. (1942) demonstrated that high temperature (33°C) inhibits the formation of lycopene in red tomatoes but they did not report any accumulation of prolycopene. Of course, it is possible that the biosynthesis of lycopene may have been inhibited farther back in the chain. More convincing evidence against the hypothesis is our observation that the double recessive (*rrtt*) is capable of synthesizing considerable quantities of prolycopene in the absence of *R*.

From a study of the hybrid between a typical red tomato (*Lycopersicon esculentum*) and a selection with a high beta-carotene content, LINCOLN and PORTER (1950) came to the conclusion that lycopene was a precursor of beta-carotene. The high beta-carotene selection had been obtained from the interspecific hybrid between *L. esculentum* and the related green-fruited wild species *L. hirsutum* (KOHLER et al. 1947). A single gene (*B*), derived from *hirsutum*, when segregating with its *esculentum* allele (*b*) gave the following percentages of beta-carotene in F_2: *bb* 0–24, *Bb*, 25–74.9, *BB* 75–100. The increase in beta-carotene content was accompanied by a corresponding decrease in lycopene. Since beta-carotene is isomeric with lycopene and differs from the latter in having ring structures at both ends, LINCOLN and PORTER assumed that lycopene was a precursor of beta-carotene.

Largely on the basis of its formula and its widespread occurrence in association with carotenoids, ZECHMEISTER and SANDOVAL (1946) concluded that phytofluene could be an intermediate in carotenogenesis. However, they were careful to point out that phytofluene and the carotenoid pigments may be derived independently from a common precursor.

PORTER and LINCOLN (1950) in summarizing work on the carotenoids presented a formal scheme for the biosynthesis of the carotenes. Starting with a nearly-saturated 40-carbon chain ($C_{40}H_{76}$), a stepwise removal of four hydro-

gen atoms at a time would lead to lycopene ($C_{40}H_{56}$). Lycopene could then be transformed to gamma-carotene by ring closure at one end and the latter into beta-carotene by ring closure at the other end. The chain up to lycopene appears logical from a chemical point of view. However, the mere presence of these supposed intermediates in many tomatoes does not demonstrate that they are in fact intermediates, since they could just as well be derived independently from common precursors. In fact their presence in ripe tomatoes, in some cases in substantial amounts, is puzzling if they are true intermediates.

On the other hand, GOODWIN and his coworkers (GARTON et al. 1951; GLOVER et al. 1951; GOODWIN and LIJINSKY 1951; GOODWIN 1952), on the basis of extensive experiments on growing *Phycomyces blakesleeanus* on media containing amino acids and sugar, have concluded that there is dehydrogenation of five-carbon units, most probably isovaleraldehyde, prior or subsequent to formation of the C_{40} polyene. Thus the carotenoids could be formed in parallel or in sequence. We ourselves favor the view that there is little or no conversion of many of the carotenoids one to another. The varying proportions of the different carotenoids would thus be due to the presence and availability of the various repeating units. MACKINNEY et al. recently obtained supporting evidence for this hypothesis. They demonstrated that it was possible to increase greatly the production of beta-carotene in a short period (5 to 20 hours) and of lycopene to a very minor extent by providing *P. blakesleeanus* with compounds theoretically capable of providing the terminal groups of the carotenoid molecule.

Since there is no good evidence that the recessive alleles at the yellow and tangerine loci block specific reactions towards the ends of the biosynthetic chain, they must either block reactions far back in the chain or in some other way influence the availability of early precursors. Of course, this assumes that each allele has a single primary action. GOODWIN's hypothesis has opened up a whole new set of possibilities. However, until we have more precise knowledge of carotenogenesis than we have at present, it seems more profitable to consider the role of these two loci in general terms. Homozygous yellow (*rr*) interferes with carotenoid biosynthesis and the interference is greater with some carotenoids than with others. Homozygous tangerine (*tt*), on the other hand, has no effect on the total carotenoid content but does alter the pathways and end products of synthesis.

SUMMARY AND CONCLUSIONS

The F_1 hybrid between yellow (*rrTT*) and tangerine (*RRtt*) tomatoes was red and the F_2 segregated 9 red : 3 yellow : 3 tangerine : 1 yellow-tangerine (*rrtt*). Dominance at both loci was considered as complete, since we could detect no difference in carotenoid content within the various classes. That is, all of the reds in the F_1 and F_2 (*RRTT, RrRR, RRTt, RrTt*) had the same carotenoids as typical red varieties; similarly the yellow (*rrTT, rrTt*), and the tangerine (*RRtt, Rrtt*) could not be distinguished from the parental types.

Substitution of *tt* in an otherwise red genotype had no effect on the total

carotenoid content but did markedly alter the individual components. Thus *tt* alters the pathway and products of synthesis.

On the other hand, *rr* interferes with the total carotenoid production. In an otherwise red background *rr* only produced about five percent of the total carotenoids characteristic of the red. Contrary to expectation the double recessive (*rrtt*) had three to four times as much total carotenoid as the yellow. As in the case of the yellow, *rr* interfered with the production of all carotenoids in the yellow-tangerine, but again the individual pigments were affected differently. Thus, the interaction at the two loci are nonadditive, both in regard to the total content and to the constituent pigments. Further data will be required before the primary action of the alleles at the yellow and tangerine loci can be determined with greater specificity.

In conclusion, all of the data conform to MACARTHUR's 9 red : 3 yellow : 4 tangerine (orange). If the yellow-tangerine, which may at times be as highly colored as the tangerine, is considered to be identical with MACARTHUR's light tangerine (light orange), there is complete agreement with the latter's 9 : 3 : 3 : 1 ratio. The failure of FLEMING and MYERS (1938) to confirm MACARTHUR's results in their F_2 generation could very well be due to a combination of variability of the environment and to segregation at genes at other loci, especially since they obtained the expected 1 : 1 ratios in their backcross generations. In any case, their elaborate explanation does not apply to the present data.

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THE INHERITANCE OF CAROTENOID PIGMENT SYSTEMS IN THE TOMATO[1]

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IN the tomato three types of fruit flesh color have long been known. The red flesh (*RRTT*) of normal canning tomatoes is due to the presence of major quantities of the pigment lycopene. A minor quantity of beta-carotene is also present, as are traces of certain other carotenoid pigments. Yellow-fleshed tomatoes (*rrTT*) are characterized by the lack of pigment in major quantities. Orange tomatoes (*RRtt*) of the Jubilee or Tangerine varieties, on the other hand, contain a mixture of carotenoids of which prolycopene and zeta-carotene are the principal components. Recently a new pigment type, orange in color, has been isolated (LINCOLN and PORTER 1950). Unlike the orange Tangerine type, this tomato contains largely beta-carotene with minor quantities of lycopene. LINCOLN and PORTER postulate that in beta-orange tomatoes the shift from red (lycopene) to orange (beta-carotene) depends primarily upon a single gene *B* which lacks dominance.

When the present study was initiated the monohybrid segregations of *R/r* and of *T/t* were well established on a visual basis (MACARTHUR 1931, 1934). Moreover, considerable analytical information was available concerning the parent pigment types (LEROSEN, WENT and ZECHMEISTER 1941; ZECHMEISTER, LEROSEN, WENT and PAULING 1941; LEROSEN and ZECHMEISTER 1942; NASH and ZSCHEILE 1945; PORTER and ZSCHEILE 1946a, 1946b). Crosses between *r* and *t*, however, had not been thoroughly analyzed, and crosses involving *B* and either *r* or *t* had not been reported. The possibility existed that *B* might be an allele of either *r* or *t*. FLEMING and MYERS (1937) cite correspondence with MACARTHUR in which the latter suggested that crosses involving Tangerine orange (*RRtt*) and yellow (*rrTT*) produce a 9 red : 3 yellow : 4 orange (3 orange and 1 light orange) F_2 ratio. From their own data, however, these authors suggested that the inheritance was considerably more complicated. Recently JENKINS and MACKINNEY (1951) have confirmed MACARTHUR'S prediction. Moreover, MACKINNEY and JENKINS (1949, 1952) have extended the analytical information with regard to parent types and have included an analysis of the segregants involving these two genes. The present work supports the data reported by MACKINNEY and JENKINS with respect to *R* and *T*, and in addition extends the analysis to include the gene *B*.

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METHODS

The characterization of the four parental pigment types, characterization of the six F_1 hybrids produced by crossing these parents in all combinations, and an analysis of the F_2 populations were undertaken in the present study. The variety Rutgers was chosen as a representative red type (*RRTT*). Jubilee, a Tangerine derivative, was chosen as representative of the Tangerine orange group (*RRtt*). For the yellow (*rrTT*) parent, a selection of PI 91458 was used. This line is characterized by the greatly reduced production of carotenoids. We have accordingly called this strain "low total." The identity of the *r* locus was proven by test crosses. For the beta-orange (*BB*) parent an advanced selection of the parent strain used by LINCOLN and PORTER (1950) in determining the inheritance of beta-carotene in crosses with the red variety Indiana Baltimore was used.

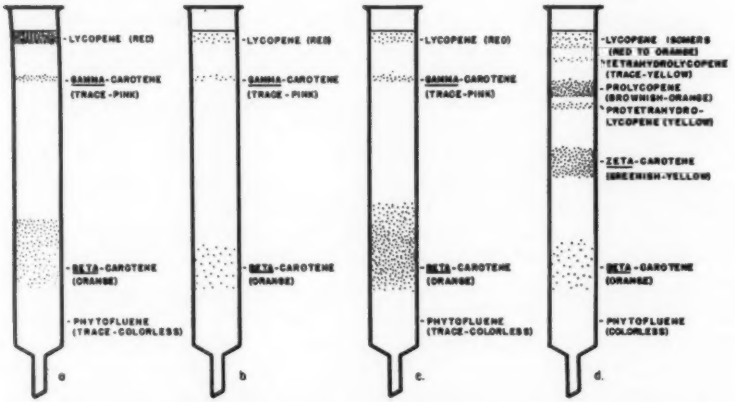

FIGURE 1.—Chromatograms of the four parent pigment complexes. a. Rutgers (red); b. low total (yellow); c. beta-orange; d. Jubilee orange.

Note: Under ultra-violet light the bands of phytofluene appear fluorescent.

Certain methods of chemical analysis used in the present work have been described in detail by ZSCHEILE and PORTER (1947), PORTER and ZSCHEILE (1945a) and PORTER and LINCOLN (1950). In brief the method was as follows:

The homogenized fruits were extracted with a mixture of acetone and hexane, the xanthophylls and esters removed, and a 20 to 25 gm aliquot of the remaining carotene solution chromatographed on magnesia. The analyst recorded the pigments present and the approximate width of each band. These data were sufficient to classify the pigment system in most cases. However, when quantitative data were desired, either of two procedures was used, depending on the complexity of the pigment system. When the chromatogram showed lycopene and beta-carotene predominating to the near exclusion of all

other pigments, direct spectrophotometric readings were made on the carotene solution and the amounts calculated. When more precise information was required or more complex pigment systems were encountered the individual pigments were eluted from the chromatogram and quantitatively determined.

It should be noted that in the method described xanthophylls and esters were discarded in the phasic separation involved in extraction. The small quantity of fruit extracted in the individual samples may also account for the fact that pigments present in extremely small quantities may escape detection entirely on the chromatographic columns.

All crosses and selfs were produced under controlled conditions in the greenhouse. In characterizing the parental strains, selfed progenies of the original plants used in the crosses were analyzed. In the F_2 populations every plant was analyzed when the original cross involved the high beta-carotene parent. In crosses involving Jubilee orange and high beta, for example, chemical characterization was the only method available for separating the two types. In populations involving segregations of the genes *R* and *T* only, the number of samples analyzed was sufficient to detect any aberrant types which visual classification might fail to detect.

THE PARENT PIGMENT TYPES

The pigment system which was typical of each of the four parent strains is illustrated in the chromatograms sketched in figure 1. (In this paper we have followed the nomenclature used by PORTER and LINCOLN 1950. Protetrahydrolycopene has been designated as psi-carotene, and tetrahydrolycopene as neurosporene by other authors.) Three of the types, red, yellow and beta-orange were differentiated by quantitative shifts involving the same pigments. Jubilee orange, on the other hand, was distinguished from the other three by possessing a number of different pigments, several of which could not be detected in any of the other parent types.

TABLE 1

Major carotenes of the fruit of 16 Jubilee orange (RRttbb)[1] tomatoes.

Pigment[2]	Mean % s
Lycopene isomers	4.2 ± 1.6
Prolycopene	36.2 ± 7.7
Protetrahydrolycopene	17.6 ± 2.7
Zeta-carotene	40.5 ± 6.8
Beta-carotene	1.8 ± 1.1

[1]This genotype was assigned after the subsequent analyses of the F_2 progenies reported in the present paper.

[2]These fruits contained a mean total carotene content of 100.1 ± 26.2 micrograms per gram fresh weight. Total carotene for Jubilee orange was calculated by the addition of the quantities obtained for the five major pigment components. Trace pigments were not included in the calculation. In addition to the pigments recorded the fruits contained 24.3 ± 10.1 micrograms/gm fresh weight of the colorless polyene phytofluene.

The quantitative relationship between the various pigments of the Jubilee complex was determined by chromatographic separation of each of the major pigment components. Table 1 presents the mean proportion of each pigment and the total pigment content as determined by addition of the quantities determined for each fraction.

In addition to the pigments assayed, Jubilee orange tomatoes contained an average of 24.3 micrograms per gram fresh weight of the colorless polyene

TABLE 2

The major carotene content of the fruit of three parent tomato strains and six F_1 hybrids.

Progeny	Year	Number of plants sampled	Mean total carotene content		Mean lycopene content		Mean beta-carotene content	
			micrograms/gm fresh wt.	s	%	s	%	s
Rutgers (red)	1949[2]	9	70.8	± 9.2	91.8	± 2.2	8.2	± 2.2
RRTTbb[1]	1950	20	88.4	± 17.0	97.0	± 2.4	3.0	± 2.4
Low total (yellow)	1949	11	1.4	± 0.2	19.7	± 12.3	80.3	± 12.3
rrTTbb	1950	20	2.2	± 0.6	38.6	± 19.3	61.4	± 19.3
Beta-orange	1949	11	71.3	± 14.6	10.5	± 2.0	89.5	± 2.0
RRTTBB	1950	19	83.6	± 16.2	6.9	± 1.8	93.1	± 1.8
F_1 Rutgers × low total	1949	10	78.0	± 19.8	98.1	± 1.9	1.9	± 1.9
RrTTbb (red)	1950	11	75.6	± 12.9	93.1	± 3.9	6.9	± 3.9
F_1 Rutgers × Jubilee	1949	10	84.4	± 21.9	95.7	± 2.3	4.3	± 2.3
RRTtbb (red)	1950	5	70.9	± 14.9	92.4	± 2.5	7.6	± 2.5
F_1 Rutgers × beta	1949	5	66.8	± 12.7	51.3	± 4.8	48.7	± 4.8
RRTTBb (orange-red)	1950	10	82.3	± 15.0	52.8	± 3.8	47.2	± 3.8
F_1 Jubilee × low total	1949	11	81.3	± 13.2	96.3	± 2.1	3.7	± 2.1
RrTtbb (red)	1950	10	97.9	± 18.1	95.3	± 2.8	4.7	± 2.8
F_1 beta × low total	1949	17	39.6	± 5.5	32.3	± 6.6	67.7	± 6.6
RrTTBb (orange-red)	1950	11	66.6	± 10.8	41.3	± 1.7	58.7	± 1.7
F_1 Jubilee × beta	1949	11	61.7	± 10.3	46.1	± 3.8	53.9	± 3.8
RRTtBb (orange-red)	1950	10	72.4	± 9.2	44.8	± 7.8	55.2	± 7.8

[1]The genotypes assigned to the various progenies are those shown to be correct by the subsequent analyses of the F_2 progenies reported in the present paper.

[2]The plots were located on the Agronomy Farm in 1949 and on the O'Neall Farm in 1950 (Lafayette, Indiana).

phytofluene. Red, and beta-orange tomatoes contained only traces of this colorless polyene. Phytofluene was present in such small quantities in yellow tomatoes that it remained undetected when small individual samples were assayed.

Red, yellow and beta-orange tomatoes contained lycopene, gamma-carotene and beta-carotene. Lycopene and beta-carotene were the two major pigment components in each system. Gamma-carotene, a structural intermediate between lycopene and beta-carotene, was present only in trace quantities in each of the three types. Table 2 presents the mean total carotene content and the

relative proportions of lycopene and beta-carotene in these three parent types and in the six F_1 hybrids produced by all possible combinations of the four parents.

The data reported in table 2 were derived by direct readings on the crude pigment extract. However, more than half of the samples were also analyzed by separation of the beta-carotene fraction. Reasonable agreement was observed between the two methods. The genotypes shown to be correct by subsequent analyses of the F_2 progenies have been supplied in tables 1 and 2.

The low total parent differed from the red and the beta-orange largely in the gross restriction of total pigment production. Both lycopene and beta-carotene were present in the yellow strain, and in the majority of cases the beta-carotene content predominated. These observations are in line with those reported by MACKINNEY and JENKINS (1949) in which, on the basis of bulk samples, yellow lines were shown to contain both lycopene and beta-carotene, with beta-carotene representing the major fraction.

A consideration of the data presented in tables 1 and 2 shows that the substitution of the genes *rr* in place of *RR* resulted in the restriction of pigment production. The substitution of *tt* for *TT* resulted in the production of pigments of the Jubilee complex, principally zeta-carotene and prolycopene. *TT* plants produced mainly lycopene and beta-carotene. In a lycopene, beta-carotene system, the substitution of *BB* for *bb* reversed the relative proportions of lycopene and beta-carotene resulting in a plant which produced major proportions of beta-carotene.

THE PIGMENT SYSTEMS OF THE F_1 HYBRIDS

With respect to the six F_1 hybrids, all produced a pigment system containing lycopene, traces of gamma-carotene, and beta-carotene. In addition to these

TABLE 3

The F_2 populations derived from crosses involving the four parent pigment types.

Cross	Total no. of plants	Chemical classification number of plants						Chemical ratio	χ^2
		Beta-orange	Orange-red	Red	Jubilee-orange	Yellow	Pale orange		
F_2-Rutgers × low total *RRTTbb* × *rrTTbb* (red × yellow)	273			196		77		3:1	1.4957
F_2-Rutgers × Jubilee *RRTTbb* × *RRttbb* (red × orange)	191			150	41			3:1	1.2722
F_2-Jubilee × low total *RRttbb* × *rrTTbb* (orange × yellow)	147			84	25	30	8	9:3:3:1	.6337
F_2-beta × low total *RRTTBB* × *rrTTbb* (orange × yellow)	200	40	70	36		54		3:6:3:4	.880
F_2-Jubilee × beta *RRttbb* × *RRTTBB* (orange × orange)	237	45	85	42	65			3:6:3:4	.949

pigments, all produced traces of phytofluene. Three of the F_1 hybrids produced chromatograms which were indistinguishable from that produced by the red parent. The remaining three produced chromatograms which were intermediate between those produced by the red parent and the beta-orange parent.

A comparison of the quantitative data (table 2) for the Rutgers parent (*RRTTbb*) and the F_1 hybrid of Rutgers × low total (*RrTTbb*) shows that each produced comparable quantities of total carotene and each contained approximately 95% lycopene. From this we may deduce that *R* is dominant to *r* chemically as well as visually. Comparing Rutgers with the F_1 of Rutgers × Jubilee (*RRTtbb*) shows a comparable situation and leads to the conclusion that *T* is strictly dominant to *t* both chemically and visually.

A comparison between the F_1 of Rutgers × beta (*RRTTBb*) and either the Rutgers parent (*RRTTbb*), or the beta parent (*RRTTBB*), suggests that the gene *B* lacks dominance. The heterozygote produced quantities of lycopene and beta-carotene intermediate between those produced by the two parents. The visual appearance of such fruit was also intermediate between the two parents, the flesh color being orange-red.

THE F_2 POPULATIONS

The analyses of the F_2 populations are summarized in table 3. From the standpoint of new pigment types, only one system was found which has not been discussed under the parent and F_1 types. This new type, a pale orange, was typical of the *rrtt* genotype (see below).

The F_2's of the crosses of Rutgers (*RRTTbb*) × low total (*rrTTbb*) and of Rutgers × Jubilee (*RRttbb*) yielded the 3 to 1 ratios expected. Chemically the plants in the progenies were shown to produce pigment systems identical with the parent types.

The one new pigment system produced was the result of recombination of *r* and *t* in the F_2 of the cross of Jubilee (*RRttbb*) × low total (*rrTTbb*). Chemically this cross produced the four classes expected in a dihybrid ratio. The double recessive *rrtt* produced a pigment system in which the amount of pigment was restricted, as might be expected on the basis of *rr*. The pigments which were produced were of the Jubilee type, as might be expected on the basis of *tt*. Unlike the Jubilee parent, however, zeta-carotene was produced in only limited quantities. Prolycopene was by far the major constituent. Table 4 presents a detailed analysis of bulk samples of three *rrtt* plants. As has been recently reported by MACKINNEY and JENKINS (1952), the *rrtt* genotype produced more total carotene than did *rrTT* plants, although the total carotene production was still quite limited as compared with any genotype carrying dominant *R*. The genotypes of the three plants on which data were collected were proven by test crosses to both the low total and the Jubilee parent.

In table 3 it may be noted that the F_2 of the cross of Rutgers (*RRTTbb*) × beta (*RRTTBB*) is not given. This progeny was not analyzed in the present study. The original report by LINCOLN and PORTER (1950) was a product of this laboratory. Moreover, careful consideration of the segregations in the

remaining two crosses in table 3 lends support to their data. In the F_2 of the cross of beta (*RRTTBB*) × low total (*rrTTbb*) the ratio obtained was 3 orange : 6 orange-red : 3 red : 4 yellow. Of the yellow plants in the F_2 population, those carrying *BB*, *Bb* or *bb* could not be distinguished phenotypically. Nor was any chemical distinction observable with small individual samples. That certain of these yellow fleshed plants actually contained *B* in a masked condition was shown by crossing 10 of the F_2 yellows by the beta parent (*BB*). Among the 10 crossed, progenies of the three types expected were obtained proving that certain F_2 yellows were *BB*, *Bb* and *bb* respectively.

In the highly pigmented portion of the population (*R*–), the plants fell into the 1 : 2 : 1 ratio typical of the F_2 segregation of *Bb*. The class limits used were those proposed by LINCOLN and PORTER (1950). The 146 highly pigmented plants gave a complete range of beta-carotene values. The distribution curve showed three distinct peaks corresponding to values typical of a red, a beta

TABLE 4

The quantities and relative proportions of pigments and phytofluene in bulk samples of three plants of the rrtt genotype.

Plant No.	23		44		50	
Pigment or polyene	Micrograms/gm fresh wt.	Pigment %	Micrograms/gm fresh wt.	Pigment %	Micrograms/gm fresh wt.	Pigment %
Lycopene isomers	2.72	23.3	2.27	24.6	1.62	29.6
Prolycopene	7.73	66.2	5.92	64.1	2.72	49.7
Protetrahydro-lycopene	.48	4.1	.39	4.2	.38	6.9
Zeta-carotene	.34	2.9	.47	5.1	.32	5.9
Beta-carotene	.40	3.4	.19	2.1	.43	7.8
Phytofluene	.48		.30		.39	
Total carotene[1]	11.67		9.24		5.47	

[1]Derived by addition of pigment fractions.

parent, and the F_1 of a cross between these two types. The low points on the distribution curve corresponded to the low points reported by LINCOLN and PORTER in their report of a cross involving a red variety with a beta type.

We may deduce from this cross that *B* is not an allele of *R*. Dominant *R* is required for the production of pigment in quantity regardless of the constitution with respect to *B*. In the presence of *R*–, the gene *B* appears to behave as originally postulated. In the presence of *rr*, the gene *B* is without apparent effect.

A somewhat comparable situation holds for the last F_2 reported in table 3. The ratio chemically was 3 orange, beta : 6 orange-red, intermediate : 3 red, lycopene : 4 orange, Jubilee. In this case, however, the beta-orange type could not be distinguished visually from the Jubilee orange type. The visual ratio thus became 7 orange : 6 orange-red : 3 red. Plants carrying *tt* produced pigment systems comparable to the Jubilee parent regardless of the genotype with respect to *B*. In the presence of *T*–, again we obtained the 1 : 2 : 1 segregation of *Bb*,

yielding the orange, beta type; the orange-red, intermediate type; and the red, lycopene type. From these F_2 data we may conclude that *B* is not an allele of *T*. *T* must be dominant before *B* can be expressed and *tt* produces pigments of the Jubilee complex regardless of the genotype at the *B* locus.

DISCUSSION

It is apparent that the major pigment shifts reported above depend upon the action and interaction of at least three independent loci, *R*, *T* and *B*. Since these genes control radically different pigment systems, they merit consideration as to their probable role in pigment synthesis.

Those who have investigated plants carrying *rr* from a chemical standpoint have agreed that *R* favors pigment synthesis, while *rr* produces only a limited amount of pigment. Beyond this point, however, several suggestions have been made regarding the role of *R*. LeRosen et al. (1941) reported that *rr* genotypes produced no lycopene. They suggested that *R* determined first the presence of this pigment. Zechmeister et al. (1941) suggested that the gene *R* might be involved in the synthesis of prolycopene in both Tangerine orange and red tomatoes and that the gene *T* might be responsible for the conversion of prolycopene to lycopene in red tomatoes. Lincoln and Porter (1950) apparently believed that *R* possessed a certain specificity for lycopene formation for they placed *R* in their synthetic scheme between zeta-carotene and lycopene.

With the knowledge of the *rrtt* genotype, Mackinney and Jenkins (1952) state that *R* clearly favors carotenoid synthesis, and that when *T* is recessive, prolycopene is synthesized regardless of *R*. From this, and from the present work, it is clear that *R* has no specificity for lycopene production, or for prolycopene or zeta-carotene production. *R–T–* produces a lycopene, beta-carotene system; *rrT–* produces the same qualitative system in limited quantity. *R–tt* produces the Jubilee or Tangerine system; *rrtt* produces the same qualitative system in limited quantity. The simplest explanation of the role of *R* would place *R* early in the sequence of synthesis. *R* appears to be responsible for the production of an unknown precursor (or precursors) in quantity.

Two difficulties are encountered with this view. The data suggest that *rrtt* produced more pigment than *rrTT*. Mackinney and Jenkins (1952) have reported a similar situation in unrelated material. Thus, if *rr* were responsible for the production of a limited amount of precursor, the end products of which were determined by the alternate alleles at the *T/t* locus, it would be reasonable to expect that the total carotene production in each case should be similar. Since we have discarded the oxygen derivatives in the extraction procedure, it is possible that both might produce comparable yields if all carotenoid derivatives were taken into consideration.

The second difficulty encountered with the view that *R* and *r* control merely quantitative reactions is the fact that the relative proportions of lycopene and beta-carotene were altered in the *rrT–* genotypes as compared with the *R–T–* genotypes. Similarly, the relative proportions of zeta-carotene and prolycopene

were altered in the *rrtt* genotypes as compared with the *R–tt* genotypes. The significance of these pigment shifts, however, is obscured by the fact that the primary action of *rr* appears to be the gross restriction of pigment synthesis. In view of this, the most plausible role of *R* and *r* would seem to be purely quantitative as regards precursors. This view is strengthened by the fact that neither *rrTT* nor *rrtt* fruits contained known polyene components which distinguished them from plants carrying dominant *R*, nor was any component produced in greater quantity in the recessive *r* genotypes.

With regard to the action of gene *T*, it seems to be generally agreed that *T* converts pigments of the Jubilee complex into a lycopene, beta-carotene system. There is a question as to which pigment is typical of the Jubilee complex, or which pigment serves as an immediate precursor for lycopene. ZECHMEISTER and WENT (1948) favor prolycopene. PORTER and LINCOLN (1950) favor zeta-carotene. If the proposed structures of zeta-carotene and prolycopene are correct, it is incompatible with modern genetic theory to assume that a single gene *T* may mediate the conversion of both zeta-carotene and prolycopene to lycopene. In the case of zeta-carotene a dehydrogenation is required to produce lycopene. With prolycopene a shift of bonds from the cis to trans configuration is required.

The above-mentioned authors lacked a knowledge of the *rrtt* genotype when these opinions were expressed. Plants of this genotype produced prolycopene in fair quantity (table 4), but the relative proportion of zeta-carotene was decreased as compared with *RRtt* plants (table 1). This may suggest that prolycopene rather than zeta-carotene is the key compound in *tt* genotypes. If ZECHMEISTER and WENT'S (1948) contention that the action of the gene *T* is one of stereochemical guidance is correct, *T* determines the spatial configuration of the molecule with respect to cis and trans bonds. On this basis *T* is not responsible for a dehydrogenation process which converts zeta-carotene to lycopene as postulated by PORTER and LINCOLN (1950). The saturated carotenes and polyenes might result from side reactions in which carotene acts as a suitable hydrogen acceptor.

The dominance relation of the gene *T* would seem to infer that some component of the Jubilee complex is converted into either lycopene or beta-carotene. Whether this step is one of dehydrogenation or of spatial direction cannot be answered at the present time. Moreover, in the tomato, the fact that phytofluene is present in much larger quantities in the Jubilee system than in the red, yellow or beta-orange types fails to prove more than coexistence of phytofluene with the more saturated carotene pigments. From a genetic standpoint, proof is still lacking that phytofluene is an immediate precursor for carotene, as has been suggested by ZECHMEISTER (1948) and PORTER and LINCOLN (1950).

Where lycopene and beta-carotene are present in quantity, the relative proportion of lycopene or beta-carotene is determined by the alternate states of *B/b*. The fact that gamma-carotene is always present in trace amounts in these systems suggests an interconversion of lycopene to beta-carotene, or *vice versa*,

mediated by the action of *B* or *b*. If, as is suggested by the data, *B* lacks dominance, this gene is of little assistance in determining the direction of synthesis. Lycopene may be formed first as PORTER and LINCOLN have suggested, or beta-carotene may appear first in the sequence.

Before concluding the discussion it should be noted that there is considerable evidence from advanced breeding lines that the *B* gene may be modified by a dominant inhibitor contributed by certain other tomato strains. While the hypothesis remains to be proven, certain aberrant results suggest that *B* may actually be a dominant gene and that the intermediate nature of the F_1, as well as the F_2 segregations of *Bb*, might be accounted for if the beta-orange parent lacked a dominant inhibitor of *B* contributed by the other parent. Among the progenies derived from crosses involving orange (beta-carotene) selections and red varieties are certain lines which breed true for orange-red color and consistently yield intermediate beta-carotene values. Moreover, some advanced selections which are high in beta-carotene content yield progenies of two types, those breeding true for high beta-carotene content, and those segregating both orange (beta) and red (lycopene), but lacking intermediate orange-red plants. A further analysis of the *B* gene is in progress.

SUMMARY

Four true breeding types of tomatoes are known which differ with respect to the carotenoid pigments produced in the flesh of the fruit. Three of these, the red (*RRTTbb*), the yellow (*rrTTbb*) and the beta-orange (*RRTTBB*), are characterized by quantitative shifts which involve the pigments lycopene and beta-carotene. The fourth type, Jubilee orange (*RRttbb*), possesses a different pigment system in which zeta-carotene and prolycopene are the major components. By analyzing the four parent types, the six F_1 hybrids produced by crossing these parents in all combinations, and the F_2 progenies derived therefrom, it is shown that these pigment systems depend upon the action of three independent genes *R*, *T* and *B*. *R* and *T* are shown to be dominant chemically as well as visually. The data presented with regard to *B* suggest that this gene lacks dominance. The primary action of gene *R* appears to be the production of an unknown precursor in quantity. *T* converts pigments of the Jubilee system into a lycopene–beta-carotene system, and *B* determines the relative proportions of lycopene and beta-carotene in the presence of *R* and *T*. Certain difficulties encountered in accepting this view with regard to the action of *R* are noted. These arise primarily from the consideration of the quantities of pigments and the relative proportions of certain pigments produced by the genotypes *rrTT* and *rrtt* as compared with *RRTT* and *RRtt*.

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THE EFFECT OF BORON ENRICHMENT ON SLOW NEUTRON-IRRADIATED TISSUES[1]

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THE biological effects of thermal neutrons are due to the ionizing radiations emitted when atoms of the irradiated tissue capture these neutrons. The compound atomic nucleus formed by neutron capture is unstable and almost immediately emits "capture" radiation to form a new isotope which may be stable, or unstable and subject to decay by the emission of radiation. In a previous study (CONGER and GILES 1950) on the production of chromosomal aberrations in the flowering plant Tradescantia by thermal neutrons (very slow neutrons with an average energy of 0.025 ev) from the Oak Ridge nuclear reactor, it was found that 99 percent of the total ionization from capture reactions in the Tradescantia tissue was due to only three elements, hydrogen, nitrogen and boron. These emit a gamma ray, a proton, and an alpha particle respectively. Boron alone was responsible for 30 percent of the ionization absorbed in tissue which, at first glance, seemed surprising since boron is present in only 2.9 ppm of the wet weight of this tissue. However, two factors conspire to make boron so important in its contribution to the total ionization from capture in spite of its insignificant chemical concentration. The first is its very high capture cross section which is about 400 times that of most of the other biological elements (The capture cross section of an isotope is the area, in cm^2, effectively presented by a single nucleus of the element to an incident beam of neutrons, i.e., its affinity for neutrons.); the second is the fact that boron emits an alpha particle whose range is only about 12 μ in tissue. Thus all its energy is usually absorbed within the same cell in which it is produced. The reaction involved is

$$B^{10} + \text{neutron} \longrightarrow [B^{11}]^* \longrightarrow Li^7 + 2.40 \text{ mev alpha particle.}$$

The importance of boron in these reactions suggested that a further study could be made by increasing the boron content of the tissue, thereby increasing the proportional amount of effect due to boron and the total effect due to a unit dose of thermal neutrons. This increase was attained in two ways: by increasing the boron content of the tissue, and by utilizing the separated stable B^{10} isotope which has a cross section of 3800×10^{-24} cm^2, about five times that of the natural mixture of B^{10} and B^{11} isotopes (715×10^{-24} cm^2).

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METHODS AND RESULTS

Boron enrichment of the plants was accompanied by growing in two glass jars six plants of *Tradescantia paludosa* ANDERSON and WOODSON (Sax Clone 5) with the roots immersed in a boric acid solution of 10 ppm of boron. The plants had been removed from pots, the roots washed free of soil, all inflorescences picked off, and then grown in the boric acid solution in the greenhouse for about one and one-half months before they were used. By this time, plants were somewhat chlorotic but microspore development in the nonsterile buds seemed normal; all inflorescences subsequently irradiated had their complete development in the boric acid solution. The boric acid was especially prepared by DR. PAUL TOMPKINS (1949) from a sample of 90 percent pure B^{10}. Inflorescences were picked from the plants on the day of use and divided at random into two groups. The first group was prepared for a spectrographic analysis of the boron content of the anthers as follows: anthers were removed from about 100 buds, selected at about the same stage of development as the irradiated buds, dried in an oven, ashed, and analyzed spectrographically for boron. The boron analyses were made by DR. CYRUS FELDMAN of this laboratory. The remaining inflorescences were irradiated in the biological thermal neutron treatment facility in the Oak Ridge nuclear reactor. The physical characteristics of the radiation beam, methods of exposure, and dose measurement have been described in a previous paper (CONGER and GILES 1950). Aceto-carmine smear preparations were made from inflorescences 22–24 hours after neutron exposure, and the frequency of induced chromatid aberrations determined from analysis of the microspores at metaphase.

In the first experiment, boron-enriched and nonenriched normal inflorescences were exposed simultaneously side by side. The aberration yield of the normal inflorescences is quantitatively the same in this experiment as those described in the previous paper, indicating that physical characteristics and neutron dosimetry are the same. The results obtained previously with nonenriched plants can be compared quantitatively with the results shown here for boron-enriched material. The data are given in table 1.

It was found that the variation in frequency of chromatid aberrations from bud to bud in the enriched inflorescences was unusually high, as much as fourfold in some cases, and was probably due to individual variation in boron content. This variation in amount of effect at the same dose made it difficult to estimate the mean aberration frequency for all the different buds. An estimate was made by taking the average of the mean frequencies of all buds at a particular dose, thus giving each bud the same weight in the average, regardless of the total number of aberrations scored from each. The simultaneously exposed nonenriched inflorescences demonstrated the usual variation in aberration frequency from bud to bud, about $\pm$ 10 per cent, and for these the mean frequency is the average of the total number of aberrations observed.

The rate of aberration production per unit dose of thermal neutrons was estimated by fitting a weighted least squares linear regression line, each experimental point being weighted by the number of buds involved in the mean

value. The dose-aberration rate for control plants are taken from the more extensive data of the previous paper which fits the control data from this experiment.

It seems natural to assume that the observed difference in effect between enriched and nonenriched buds was due entirely to the greater amount of alpha radiation resulting from the higher boron concentration in enriched buds.

TABLE 1

Frequency of 22- to 24-hour chromatid aberrations from thermal neutron irradiation of normal and boron-enriched plants.

Experiment	Expos. time (mins.)	Dose (thermal neutrons per cm^2) ($\times 10^{-11}$)	No. of buds	Total no. of cells	Aberrations					
					Isochromatid		Chromatid plus isocd.		Chromatid chromatid interchanges	
					No.	Mean cell*	No.	Mean cell*	No.	Mean cell*
SN 16, 1 June '49	1	0.38	4	356	30	0.11	61	0.22	14	0.043
31 ppm boron	1	0.65	5	309	46	0.17	91	0.34	24	0.11
	2	1.3	6	236	141	0.60	240	0.98	55	0.28
	3	1.9	3	97	52	0.51	101	1.0	21	0.22
	4	2.7	2	46	78	1.7	135	2.6	41	0.89
	5	3.5	3	56	64	1.3	131	2.8	46	1.2
	6	4.0	2	80	81	1.1	151	2.0	33	0.41
SN 17, 6 July '49	1	0.50	10	265	110	0.48	173	0.72	45	0.20
91 ppm boron	2	1.0	7	157	79	0.71	136	1.2	28	0.28
	4	1.8	6	125	83	0.83	152	1.5	49	0.51
SN 16, 1 June '49	1	0.38	1	100	4	0.04	10	0.10	2	0.02
control	1	0.65	2	132	8	0.06	25	0.19	2	0.015
(not enriched)	3	1.9	3	162	38	0.23	72	0.44	20	0.12
	4	2.7	2	131	37	0.28	61	0.46	11	0.084

	Aberration production Aberrations per cell per 1×10^{11} n/cm^2		
	Isochromatid	Chromatid plus isocd.	Chromatid chromatid interchanges
Boron enriched SN 16 & 17 combined	0.416	0.75	0.242
Control (not enriched)	0.107	0.226	0.044

*Mean/cell is the average of the means of the individual buds for the boron-enriched buds; for normal control buds, it is the mean of the total number of aberrations.

However, it is possible that such an increase in boron might make the cells physiologically more sensitive to radiation, independent of the alpha radiation involved. To eliminate the possibility that an increased "physiological radiosensitivity" caused the observed difference, inflorescences from boron plants and normal plants were exposed simultaneously to X-rays. The results are given in table 2.

There is no difference in the response of the normal and boron-enriched buds to X-rays; apparently, the difference observed from thermal neutron

irradiation is not due to a change in "physiological radiosensitivity" but rather to increased alpha radiation in the enriched plants.

DISCUSSION

It is apparent from the comparison of aberration yields in table 1 that boron enrichment has increased the effect per unit dose of thermal neutrons by about four or five times. How well this agrees with the anticipated increase in effect may be estimated on a physical basis from a consideration of the two changes which have been made in the boron-enriched plants. These two changes are the increase in the over-all concentration and the increase in the effective capture cross section (per atom of boron) by using the B^{10} isotope. A comparison of the anticipated and observed ratio of effect is made in table 3. Data on the ionization from a unit dose of neutrons are from table 8 of the previous experiment (CONGER and GILES 1950).

TABLE 2

Yield of 22- to 24-hour chromatid aberrations from X irradiation of normal and boron-enriched plants.

X-ray dose (r)	Type of plant	No. of buds	Total no. of cells	Aberrations: Isochromatid No.	Per cell ± S.E.	Chromatid plus isocd. No.	Per cell ± S.E.	c/c interchanges No.	Per cell ± S.E.
150	Boron-enriched	4	175	171	0.980 ± 0.075	389	2.22 ± 0.11	120	0.68 ± 0.063
	Normal	3	125	135	1.08 ± 0.093	259	2.07 ± 0.13	82	0.66 ± 0.072
	Diff. ± S.E.			diff. −0.10	± 0.12		0.15 ± 0.17		0.02 ± 0.10
100	Boron-enriched	5	210	94	0.45 ± 0.046	224	1.07 ± 0.071	53	0.25 ± 0.035
	Normal	5	350	166	0.47 ± 0.037	385	1.10 ± 0.056	84	0.24 ± 0.026
	Diff. ± S.E.			diff. 0.02	± 0.059		−0.03 ± 0.09		0.01 ± 0.044

250 kvp, 15 ma X rays, 3 mm Al inherent filtration. 150 r/min. The boron-enriched and normal inflorescences at each dosage were irradiated simultaneously.

A boron enrichment of 21 times and a change in boron cross section of 4.8 times causes a total increase in boron ionization of 101 times. However, it would not be expected that the over-all effect, chromosome aberrations, would be increased 101-fold, since boron accounts for only a fraction of the total ionization generated in the tissue. The chromosomes, however, are broken by the sum of the ionization from all sources, and the amount of ionization from all other sources (gamma-ray contamination, plus H, N and Cl capture reactions) is unaffected by the change in boron. Actually, the total ionization is increased only sixfold. The ratio of effect in enriched and normal plants is not simply in the ratio of total ionization, however, because a large share of this increase is being caused by alpha particles. It is well known that for equal ionization alpha particles cause more chromosomal aberrations than do X or gamma rays; in this material, the relative biological efficiency of alpha particles is about 4.1/1 (KOTVAL and GRAY 1947). The total increase in biologically effective dose is therefore the product of ionization dose times the relative biological efficiencies of the radiations involved. On this basis, it appears that the

final result of a 101-fold increase in boron ionization is an increase in the biologically effective dose of about 18 times. The observed increase in chromosomal aberration production of from four to five times is the same order of magnitude and, considering the variables involved, is in fair agreement for an estimate of this sort. However, the fact that observed effect is less than expected is in the proper direction to satisfy a number of specific biological conditions that were noticed in this experiment which would cause just such a deviation. Judging from the variation in aberration frequency from bud to bud at the same neutron dose, it seems clear that boron concentration may vary between buds by as much as four times—in spite of the long growth period of entire plants in boric acid and the fact that all irradiated inflorescences had undergone their complete growth in the boron environment. Unfortunately,

TABLE 3

Expected and observed ratio of effect from thermal neutron irradiation of normal and boron enriched plants.

Sample	Boron		Expected				Observed		
			Physical dose per 1×10^{11} n/cm²		Biological equivalent dose per 1×10^{11} n/cm²		Aberration production Aberrations per cell per 1×10^{11} n/cm²		
	p.p.m. of wet wt. of anthers	Effective capture cross section $\times 10^{-24}$cm²	Total R.E.P.	% from B	Total[2] R.E. biol.	% from B	Isochromatid deletions	Chromatid plus isocd.	c/c interchanges
Boron enriched (av. of Expts. 16 and 17)	61	3420[1]	62.2	85	228	95	0.416	0.75	0.242
Normal	2.9	715	9.75	5.4	12.7	17	0.107	0.226	0.044
Ratio enriched/normal	21	4.8	6.4		18		3.9	3.3	5.5

[1] 90% B^{10} at 3800 + 10% B^{11} at <0.05 = 3420.
[2] Roentgen equivalent biological, when the biological efficiency of: protons = 2.6; alpha = 4.1.

analyses of the boron content of single buds could not be made; the amount was too small. A fair amount of anther sterility was found in the buds from boron-enriched plants, and because of the well-known toxic effect of high levels of boron, it is believed that the buds with sterile anthers were those with the higher boron concentration. A further concentration in boron content (on wet weight basis) in the sterile anthers was caused by the considerable amount they dried down. It is certain that a portion of the sterile anthers was unavoidably included in the spectrographic sample, but sterile anthers were, of course, automatically eliminated from the sample used for aberration analysis. Finally, it is obvious that any change in the isotopic concentration of B^{10} will have to be a decrease, caused by dilution with the normal B^{10} and B^{11} isotopic mixture in the plants and glassware. It is therefore concluded that the average boron content of the anther sample analyzed spectrographically was

higher than the sample analyzed biologically. Since the physical and chemical data of table 3 cannot allow for such an error, the expected ratio of effect given in table 3 should, in fact, be larger than the observed, as was found.

The unique radiological features of the boron neutron capture reaction in tissue suggest its use for radiation therapy. The very high capture cross section of boron, the high ionization density and very short range (about 12 μ) of the emitted alpha particle make possible the generation of very localized and large ionization doses in boron containing tissues subjected to thermal neutron irradiation. Lithium would have the same properties for this purpose as boron. The differential in ionization distribution is mainly a function of the differential in boron or lithium distribution. ZAHL et al. (1940, 1941) in a series of papers have discussed thoroughly the possibility of and suitable methods for the radiotherapeutic use of boron and lithium capture reactions, and KRUGER (1949) has demonstrated a reduction in " takes " with slow neutron irradiated mouse tumors previously soaked in boron.

SUMMARY

An increase in the amount of biological effect (chromatid aberrations in the plant Tradescantia) produced by a unit dose of thermal neutrons has been caused by an enrichment of the boron content of the tissue. The separated stable isotope B^{10} was used for enrichment because of its high capture cross section for thermal neutrons. The increase in effect is due to the alpha rays from the $B^{10}(n, \alpha)Li^7$ reaction. For the same neutron dose, the enriched plants showed four to five times as many chromatid aberrations as the nonenriched normal plants. On the basis of the boron enrichment, measured chemically, a somewhat greater increase was expected. Disagreement was probably due to nonsurvival of the flower buds with higher boron content, which were included in the chemical sample, but excluded from observation in the biological sample because of death.

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THE DELAYED APPEARANCE OF RADIATION-INDUCED GENETIC CHANGE IN BACTERIA

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FOR the bulk of the radiation-induced mutations of *Escherichia coli* to phage T1 resistance to appear as phenotypic changes, the irradiated cells must pass through a period of metabolic activity. It has been estimated that approximately thirteen cell generations occur before all of the mutations are expressed (DEMEREC 1946; DEMEREC and LATARJET 1946). The precise nature of this very considerable delay is still a matter of controversy.

Four effects might contribute to it: (1) gene mutation may not occur until some time after irradiation; (2) if there is more than one nucleus (or gene complement) in a cell at the time of mutation, cell division and the consequent nuclear (or gene) segregation may be necessary before the mutant gene can alter the phenotype; (3) a mutant gene may require a period of metabolic activity before it can change the phenotype; and (4) there may be an irregularity in the onset of division after irradiation such that many cells do not divide until an appreciable population increase has taken place, and are thus delayed in expressing their mutations. These possible effects will be termed *mutation delay, segregation delay, phenotypic delay,* and *irregularity delay.*

Evidence as to the probable magnitudes of the mutation and segregation delays has been obtained from a study of sectoring in colonies from irradiated bacteria using changes in colour response on mannitol-tetrazolium agar, to phage resistance and to lactose non-fermentation. The extent of the phenotypic delay for mutations to phage resistance has already been determined from a study of spontaneous mutation (NEWCOMBE 1948). The present experiments indicate that the combined contributions from these three sources are inadequate to account for more than one-quarter to one-half of the estimated thirteen cell generations between irradiation and expression of all induced mutations to phage T1 resistance.

The irregularity delay undoubtedly contributes wherever there is an appreciable irregularity in onset of division. However, the extent of the contribution is difficult to assess and there is no certainty that it can account for the remainder of the estimated overall delay. It is therefore necessary to consider whether the above list includes all of the factors which might contribute.

It would seem that the only possibility not so far examined is that the estimates of overall delay may be subject to a considerable systematic error, arising perhaps out of certain peculiarities in the response of the bacteria to phage. The evidence which will be presented is compatible with this interpretation.

Special attention has been directed to the question of the mutation delay, since a knowledge of the timing of the induced mutations is fundamental to an understanding of the mechanism by which they occur.

SECTORING IN COLONIES FROM IRRADIATED CELLS

If an induced mutation were delayed beyond the first gene division, mutant and non-mutant bacteria would occur together, both being descended from the one treated nucleus. To determine whether the delayed appearance of phage-resistant mutants among the descendants of irradiated cells was the result of a mutation delay, NEWCOMBE and SCOTT (1949) examined 3,480 colonies from ultraviolet irradiated cells of *E. coli*. Two of these colonies showed induced mutations to phage T1 resistance, but neither contained sensitive cells in detectable numbers. This did not rule out the possibility that mutations might be delayed until approximately the time of the first gene division, or that some mutations might occur after the first gene division, but it showed that delays beyond the first gene division could not be frequent enough to account for the very considerable and almost universal delay in the appearance of induced mutations to phage resistance.

Colour response mutations on mannitol-tetrazolium agar

Later, colonies from irradiated cells of *E. coli* B/r were found which showed well-defined sectoring for various degrees of colour response change on mannitol-tetrazolium agar. (For photoreversal studies using these mutations see NEWCOMBE and WHITEHEAD 1951. Whole-colony mutants were also observed but because some of the changes were slight these could not be scored with the same degree of accuracy as the sectored colonies.) The sectored colonies were at first believed to be derived from cells which had more than one nucleus at the time of irradiation, and to test this interpretation a selection technique was used which, it was believed, would permit the survival of descendants from single nuclei only. Irradiated suspensions were plated on mannitol-tetrazolium agar and, after a preliminary incubation period to permit expression of any mutations to phage resistance, were sprayed with a mixture of phages T1 and T5 and incubated further until colonies appeared (for details of the spray technique see DEMEREC 1946). Since these lines supposedly stemmed from single nuclei in which there had been radiation-induced mutations to phage resistance, the colonies were expected to be homogeneous for any other induced changes including the variations in colour response on the mannitol-tetrazolium indicator medium. Contrary to expectation, a proportion of the colonies were sectored for colour changes. (For a preliminary account see NEWCOMBE 1951.) Similar results were obtained with ultraviolet- and with gamma-irradiated materials (see table 1). It was also shown that streptomycin could replace phage as the selective agent.

The sectoring which survives selection could be due to: (1) delayed gene mutation; (2) genetic interchange between two nuclei (or gene complements) in the irradiated cell; (3) mutation in incompletely split chromosomes; or

TABLE 1

The effect of phage selection on the number of radiation-induced colour variations on mannitol-tetrazolium agar, appearing as sectored colonies.

Treatment and dose (ergs per mm², or r)	Unselected			Selected[1]		
	Colonies examined	Sectored colonies	Percent sectored	Colonies examined	Sectored colonies	Percent sectored
UV 500	648	94	14.5	1688	162	9.6
UV 1,000	1027	193	18.8	957	81	8.5
Gamma 40,000	373	22	5.9	948	20	2.0
Gamma 80,000	1320	89	6.7	2448	110	4.5

[1] Plates were inoculated with 10^6 to 10^8 survivors and sprayed with a mixture of phages T1 and T5 after a preliminary incubation of 4 to 5 hours.

(4) mutation in hereditary particles other than the chromosomal genes. These possibilities will be considered in detail later. (Similar experiments were carried out independently by WITKIN (1951a), using ultraviolet, but not gamma radiation, and she has drawn essentially the above conclusions from her results.)

It should be noted that since a proportion of the mutant colonies from both the non-selection and the selection experiments were wholly mutant for colour response, a delay in induced gene mutation cannot extend beyond the first gene division in all cases.

It would not be surprising if ultraviolet-induced mutations were appreciably delayed, since *E. coli* does not become insensitive to photoreversal until there has been a half an hour or so of metabolic activity following the irradiation (unpublished result, see also NEWCOMBE and WHITEHEAD 1951). Until the present experiments, however, there has been nothing to suggest that gamma- (or X-ray-) induced gene changes were similarly delayed.

TABLE 2

Proportions of ultraviolet-induced mutations to phage T1 resistance, appearing as wholly mutant colonies and as sectored colonies.

Method	Dose (ergs per mm²)	Colonies examined	Mutant colonies whole : sectored	References
"Culture"	1,600	3,480	2 : 0	Newcombe and Scott
"Cross-streak"	500	10,000	1 : 0	Present
"Cross-streak"	2,000	10,000	0 : 2	Present
"Peel-spray"	2,000	28,000	1 : 1	Present
"Replica-plating"	4,000	18,000	2 : 1	Visconti[1]
Totals		69,480	6 : 4	

Mutations per 10^4 survivors = 1.46

[1] Personal communication.

Note: As the "peel-spray" technique relies on the appearance of the mutant portion of a colony, there might be some in which the sectoring is not sufficiently clear for detection and which would be erroneously scored as "whole." However, the other techniques do not suffer from this defect and the total ratio cannot be appreciably biased.

Mutations to phage resistance

As it was just conceivable that the colour response variations were of different origin from the mutations to phage T1 resistance (although in both cases the changes appeared to be stable in subculture) it was decided to reinvestigate the possibility that some of the induced mutations to phage resistance might appear as colonies containing both sensitive and resistant sectors. Independently, VISCONTI (personal communication) carried out a similar study, and his results are included in table 2 together with those of NEWCOMBE and SCOTT (1949) and those from the present experiments.

In all, three techniques were used. First, suspensions of the colonies from irradiated cells were cross-streaked with phage on E.M.B. (eosin-methylene-blue) agar without any sugar. This enabled mixtures of resistant and sensitive cells to be distinguished from resistant cells alone, since incomplete lysis of sensitive cells near the edge of the phage streak gave rise to a distinctive pink colour. Second, colonies from irradiated cells were removed from the agar by means of dry sterile filter paper which was laid on the surface and peeled off again together with the adhering colonies (repeating where necessary). The number of cells remaining on the agar was sufficient so that when these plates were sprayed with phage T1 and incubated, resistant colonies and resistant sectors regrew in their original shapes. The shapes were not blurred by subsequent growth. Third, VISCONTI's technique consisted of taking impressions of the colonies with sterile filter paper, and transferring the impressions to plates previously coated with phage. (This is essentially similar to a technique devised by LEDERBERG and LEDERBERG, 1952.) The three methods are called " cross-streak," " peel-spray," and " replica-plating " in table 2.

Of a total of ten induced mutations to phage T1 resistance, six gave wholly mutant colonies, and four sectorials. Thus mutations to phage resistance and to colour change on mannitol-tetrazolium agar are similar in that both give rise to a proportion of sectored colonies.

Mutations to lactose non-fermentation

The proportions of induced " whole-colony " and " sectored " mutants of *E. coli* B/r to lactose non-fermentation (as indicated by colour response on E.M.B.-lactose agar) were studied both with and without phage T1 selection. These experiments are closely parallel to those reported by WITKIN (1951b) and the results are in qualitative agreement. Quantitative differences, however, will be noted, and our data will be treated in a different manner.

Cultures grown for 48 hours with aeration in ANDERSON's (1946) M9 medium (using lactose, instead of glucose, as the sole carbon source) were used throughout. All induced mutant sectors were tested by restreaking on E.M.B. lactose agar, only those of a white or light pink colour being scored as mutants.

With no phage selection, approximately one-third of the ultraviolet-induced mutant colonies were " whole " and two-thirds " sectored " (a ratio not significantly different from that for mutation to phage T1 resistance); with phage

selection the ratio was approximately reversed (see table 3). While our non-selection experiments are in reasonable agreement with WITKIN's (she observed approximately equal numbers of "whole" and "sectored" colony mutants), her selection experiments gave very much higher proportions of "whole" (about 90 percent). This discrepancy does not seem to be due to any difference in the methods of scoring and is difficult to account for. However, one possibility might be suggested: in a later section it will be shown that under certain conditions the spray technique can give rise to a very considerable admixture of resistant colonies of spontaneous origin (these presumably being the result of bacterial growth continuing in the presence of the phage). These spontaneous resistant colonies would tend to be homogeneous ("whole") for induced changes to lactose non-fermentation. In our experi-

TABLE 3

The effect of phage T1 selection on the proportions of ultraviolet-induced mutations to lactose non-fermentation appearing as "whole" and as "sectored" mutant colonies. (Dose = 500 ergs per mm².)

Selection	Colonies examined	Mutant colonies		
		Whole : sectored	Percent whole	Mutations[2] per 10^4 survivors
None	145,552	86:191	31.0	19.0
Phage T1[1]	195,810	194:102	65.5	15.2
Unirradiated, no selection	135,950	7:31	....	2.8

[1] Preliminary incubation, 4–6 hours. (No differences were found between 4 and 6 hours and the data have been combined; the separate ratios were 44:25 and 150:77 respectively.)

[2] The proportion of colonies with lactose mutations (whole and sectored combined) appearing in the selection experiments will be seen to be approximately 80 percent of that in the non-selection experiments, due presumably to some being lost in the former through having arisen in different lines of descent from the phage mutations. The above data are from three experiments which individually showed reductions to 46, 59 and 84 percent, as a result of selection.

ments the ratio of "whole" to "sectored" was independent of preliminary incubation over the range from 1 to 6 hours (see tables 3 and 8) and it was assumed from this that the effect was not sufficient to have biased our results. WITKIN, on the other hand, found the ratio to vary with preliminary incubation (see her table 5) and it is less certain that bias was absent in her experiments.

Whatever the nature of the discrepancy, it should be noted that no essential differences have been uncovered in the behavior of the three kinds of mutation (mannitol, phage and lactose) used in the present study.

The possibility of delayed mutation

Critical discrimination between the four alternative interpretations of the sectoring that survives selection is not possible at the present time, and it may even be that more than one mechanism contributes.

Genetic interchange between two nuclei, for example, cannot be entirely ruled out. However, if this were the cause we would have to suppose that two-thirds of the cells have more than one nucleus (or gene complement) at the time of irradiation, and that half of these produce two recombination types (with respect to the locus used for selection and that being studied) which are viable in the presence of the selective agent, plus an assumed two which are inviable in the presence of the selective agnt. This would have to be true for the character pairs: phage resistance and colour response, phage resistance and lactose non-fermentation; and at least roughly true for streptomycin resistance and colour response, and streptomycin dependence and lactose non-fermentation. It also implies a regular and complex system of recombination, a somewhat improbable set of linkage relationships, and a higher proportion of binucleate cells than our estimates (to be described later) indicate.

(It has been suggested that an experimental test could be applied using the replica plating technique to detect the expected phage-sensitive lactose-negative class of recombinants.)

If the chromosomes were split over a part of their length at the time of irradiation, this condition could give rise to sectored colonies from selection experiments. Assuming no special tendency for certain loci to be single and others split, the ratio of "sectored" to "whole" from this cause would be

$$ab(1-b)/[(1-b)^2+ab(1-b)+1/2a^2b^2] \qquad (1)$$

where a is the factor increase in likelihood of induced mutation as the result of a locus splitting, and b is the likelihood that it will be split at the time of irradiation. The proportion of sectored colonies would be expected to reach approximately 30 percent under optimum conditions (nearly equal numbers of split and unsplit loci), this being true whether a has a value of one or of two. The limiting value of 30 percent is close to the 34 percent observed in our selection experiments (and also to the 28 percent observed by WITKIN when the short, 3-hour, preliminary incubation was used). The one improbability involved in this interpretation is that optimum conditions must be supposed to have prevailed in practically all experiments.

Mutations in self-reproducing particles other than the chromosomal genes would of course tend to produce very small mutant sectors. Also, the evidence from crossing experiments, using what appear to be similar mutants of another strain of *E. coli*, argues strongly for mutation in chromosomal genes.

For these reasons, it seems more likely that the sectoring which survives selection is the result of a delay, such that many mutations do not occur (in their final form at least) until after the gene has started to divide.

THE EXTENT OF THE MUTATION AND SEGREGATION DELAYS

Where wholly mutant colonies arise from irradiated cells it would seem that mutation has probably occurred prior to the completion of gene division and in cells with only one nucleus.

Occasional wholly mutant colonies might come from binucleate (or multinucleate) cells in which the other nucleus (or nuclei) had sustained a lethal

genetic injury. Killing, however, appears to be predominantly non-genetic in this organism (although there is no reason to assume a complete absence of genetic injury) (see DEMEREC and LATARJET 1946). Further, if induced chromosomal changes were responsible for appreciable nuclear elimination, higher doses of ultraviolet might be expected to yield higher proportions of " whole-colony " mutants, which is not what has been observed (see table 4). Nor does gamma irradiation, which is believed to be more effective in producing lethal genetic changes, yield appreciably higher proportions of " whole-colony " mutants, whether administered alone or in combination with ultraviolet (table 4).

Similarly, delayed lethal mutations, if they occurred, could convert a proportion of the colonies which would have been sectored as the result of delayed lactose mutation, into whole-colony mutants by suppression of the lactose fermenting lines of descent. Our data do not rule out the possibility, but the above

TABLE 4

The effect of differences in mutagenic treatment on the proportions of "whole" and "sectored" mutant colonies, using changes to lactose non-fermentation. (Without phage selection.)

Treatment and dose (ergs per mm², or r)	Colonies examined	Mutant colonies		
		Whole : sectored	Percent whole	Mutations per 10^4
UV 500[1]	145,552	86 : 191	31.0	19.0
UV 2,000	27,131	7 : 14	33.3	7.8
UV 4,000	42,510	9 : 19	32.2	6.7
Gamma 100,000	15,920	9 : 13	40.8	13.8
Gamma + UV 500	12,120	11 : 20	35.4	25.5

[1] From table 3.

arguments can be applied also in this case and suggest that it is at least not a gross effect.

Selective elimination of non-mutant sectors during the early growth of a sectored colony might also occur giving rise to " pseudo-whole-colony " mutants. In a few cases the lactose non-fermenting colonies had a small positive area at the center, and there were also some sectored colonies in which the wild type was represented by both successful and aborted sectors. Such cases were rare however and would have little effect on the observed ratios. Also, they suggest competition after appreciable colony growth and there is little reason to expect similar competition in the early uncrowded stages. For the sake of simplicity it will be assumed in the following discussion that bias was absent and that all of the sectoring which survived phage selection was the result of delayed gene mutation.

If p and q were the proportions of multinucleate cells, and of nuclei mutating after the first gene division, the proportion of sectorial mutants obtained in the non-selection experiments would be the sum of $q - pq$ (from delayed mutation

in uninucleate cells) and p (from multinucleate cells). From our non-selection experiments

$$q - pq + p = 2/3 \qquad (2)$$

Limiting values for p and q can be determined. From the non-selection experiments we know that neither p nor q may exceed 2/3, while from the selection experiments q cannot be less than 1/3; and by substituting in formula (2) it can be seen that p may not exceed 1/2. Thus between 1/3 and 2/3 of the mutations are delayed to give mixed mutant and non-mutant descendants from a single gene complement, and from 1/2 to perhaps all of the cells are uninucleate at the time of irradiation.

Provided there is no special tendency for phage mutations to occur earlier than lactose mutations (or vice versa), some idea of the extent of the delay can be obtained from: (1) the proportion of colonies with lactose mutations in the selection as compared with the non-selection experiments, and (2), the ratio of "whole" to "sectored" lactose mutants observed in the selection experiments. The present data for example rule out the extreme case of most of the mutations being delayed many generations because the chances of phage and lactose mutations occurring in the same lines of descent would be small and few lactose mutations would survive in the selection experiments, and because the ratio of "whole" to "sectored" mutant colonies obtained in the phage selection experiments would be expected to approach equality.

A quantitative treatment is possible. It can be shown that the ratio of "whole" to "sectored" lactose mutant colonies expected from a selection experiment is represented by the expression

$$\sum_{i=0}^{n} \left[a_i \sum_{k=0}^{i} 2^{-k} b_k \right] \Big/ \sum_{i=0}^{n} \left[2^{-i} a_i \sum_{k=i+1}^{n} b_k \right] \qquad (3)$$

where a_i is the likelihood of a phage mutation in the i^{th} generation, b_k is the likelihood of a lactose mutation in the k^{th} generation, and the n^{th} generation is the last in which a mutation can occur. (The cell prior to the first gene division is generation 0; $\Sigma a_i = \Sigma b_k = 1$.) The proportion of lactose mutations which failed to appear in the selection experiments because they had occurred in different lines of descent from the same irradiated cell would be equal to one minus the sum of the numerator and the denominator in formula 3.

By substituting (and assuming $a_k = b_k$) is can be seen that a number of alternative sets of values of a_0, a_1, a_2, etc., would yield the observed 66 percent "whole-colony" mutants. However, some of these involve a low probability of mutation in one generation followed by a high probability in the next, which seems unlikely. Accepting a value of a_0 between 1/3 and 2/3 (as indicated by formula 2), and a consistent trend with successive generations ($a_k > a_{k+1}$), the range of values is considerably narrowed. One obvious solution is $a_k = 1/2^{k+1}$ (75 percent of the mutations in generations 0 and 1, 87 percent in generations 0, 1 and 2). Other solutions would be a_0, a_1, a_2, etc., equal to .48, .27, .25, 0.0, or .53, .32, .15, 0.0; and even values of .50, .50, 0.0 provide an approximate

solution. With all of the above solutions mutation is infrequent in generation 3, and it would seem that three-quarters or more of the mutations probably occur in generations 0 and 1.

Further, if higher proportions of " whole-colony " mutants were observed in selection experiments this would seem to point unambiguously to even higher proportions of early mutations. Thus WITKIN's table 5 (which shows an increase in the proportion of whole colony mutants from 72 to 92 percent as the result of increasing the preliminary incubation from 3 to 6 hours) could be interpreted as arguing simultaneously for an extended delay in gene mutation (from the effect of varying the preliminary incubation) and for an extremely limiting delay (from the final high proportion of whole-colony mutants). This paradox might indicate either that we were wrong in assuming phage and lactose mutations to be equally delayed, or that in WITKIN's experiments there was an increasing admixture of spontaneous mutations to phage resistance with increasing preliminary incubation. The latter view is favoured because the difficulty does not arise with our own data and because, as will be shown later, there is other evidence that the resistant colonies obtained using the spray technique may, under certain conditions, contain an unexpectedly large admixture of spontaneous origin.

(Delayed phenotypic expression of induced mutations to phage resistance would, of course, have similar consequences to delayed mutation, provided it is assumed that a similarly small part of the clone is affected. WITKIN interprets her data in these terms. The assumption, however, implies a considerable difference in the ways by which spontaneous and induced mutant genes develop phenotypic expression, cf. NEWCOMBE 1948; it is also contrary to our data, and is not necessarily indicated by hers.)

It should be noted also that WITKIN's observation of a higher number of lactose mutations in selection than in non-selection experiments is not confirmed by the present data. We observed slightly fewer (80 percent, see table 3) and thus have no evidence that phage and lactose mutations are correlated events. The discrepancy might conceivably be attributed to differences in the precautions which were taken to ensure uniform exposure of all cells during irradiation (in our experiments the Petri dish was shaken mechanically many times a second so that vigorous standing waves were maintained over the surface). This discrepancy, however, does not affect the main argument, and the important conclusion to be drawn from the present data is that the mutation delay is very limited, a conclusion also reached by WITKIN from the sizes of the lactose negative sectors.

THE OVERALL DELAY IN APPEARANCE OF INDUCED MUTATIONS TO PHAGE RESISTANCE

Of the four effects which might contribute to the overall delay in appearance of induced mutations to phage resistance, the combined mutation and segregation delays probably equal one or two cell generations, and the phenotypic delay two to three (see NEWCOMBE 1948), accounting for a total delay

of from three to five generations out of an observed thirteen (or 2^{13}-fold population increase). Irregularity in the onset of division must, when it occurs, prolong the apparent delay but the extent of the contribution from this source is difficult to determine. It might play a large role if there were a correlation between delay and likelihood of mutation, and in particular if this correlation applied to part of a clone in which a nuclear disturbance operated (see WITKIN 1951b, and discussions of that paper). However, such a concept would imply that mutations are correlated with one another, and although WITKIN has some evidence of this we have failed to confirm it. Unless, as seems very unlikely, the contribution from irregularity is equivalent to the remaining 8 or more generations there is a discrepancy the nature of which is still in doubt.

Accuracy of the estimates of overall delay

Since the list of probable contributing factors seems to exhaust the possibilities, we are forced to consider whether the estimates of the overall delay are subject to some large systematic error. Evidence of a number of kinds supports this interpretation.

First, both irradiated and non-irradiated cells from saturated cultures show a high "mutation rate" during the early divisions on agar, as indicated by the spray technique (see figure 1, and for a previous report, NEWCOMBE 1948). In the case of the non-irradiated bacteria the effect is probably spurious (and it is doubtful even whether it represents a manifestation of previously accumulated mutations) since alternate growth and saturation in liquid culture does not result in a greater than normal increase in number of mutants (NEWCOMBE 1948, table 4). The parallel behavior of irradiated cells suggests that a part of the apparent high mutation rate following irradiation might also be spurious and the result of some selection artifact (the irradiation perhaps acting to prolong a response to phage which is normally characteristic of the lag period).

Second, there is a curious positive correlation between dose and the extent of the overall delay (with respect to population increase) as estimated by the spray technique (for X-ray data see DEMEREC 1946, table 4; and for ultraviolet see figure 1 of the present paper). This could perhaps be due to a greater irregularity in onset of division where the dose is high, but it is equally possible that phage selection is slowed down by irradiation of the bacteria and that this slowing persists longer with the higher doses.

Third, there is ample evidence that the number of induced mutations to phage resistance can be grossly overestimated by the spray technique. Thus, the dose-response curve shows a continued rise up to at least 7000 ergs per mm^2 (DEMEREC and LATARJET 1946), although using other techniques there is a plateau (or slight decline) with doses above 500 ergs per mm^2, this being true not only for the changes to phage resistance (see table 2) but for other mutations as well (e.g., the mannitol-tetrazolium mutations, see NEWCOMBE and WHITEHEAD 1951; and the lactose mutations, see table 4). Also, very much higher numbers of mutations are detected by the spray technique (with

the higher doses, between 6 and 200 per 10^4 survivors in DEMEREC and LATARJET'S experiments) than by the single colony isolations (1.4 per 10^4 survivors in the combined data from table 2). That the induced mutations can be overestimated by as much as a hundred fold indicates that the application of phage to irradiated bacteria sometimes fails to prevent subsequent division and spontaneous mutation, even when the bacteria have had considerable time in which to recover from the effects of the irradiation.

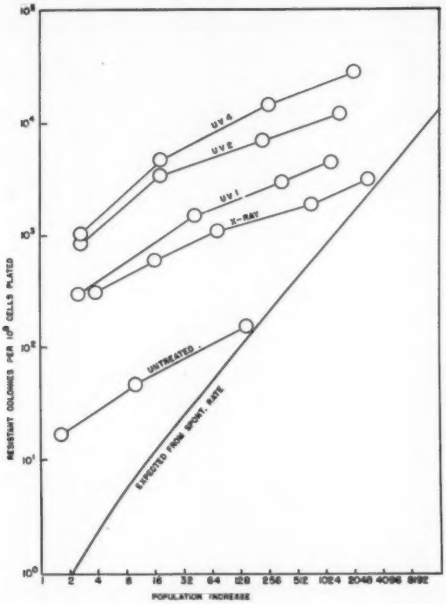

FIGURE 1.—Numbers of phage resistant colonies which result when bacteria are sprayed with phage T1 after various periods of incubation and growth on the surface of agar.

All bacteria were plated from saturated cultures; in the top four curves they were irradiated with ultraviolet light (three different doses) or with X-rays before plating. All points are averages from three or more experiments.

The precise cause of this artifact is unknown, although physiological changes in the irradiated bacteria and their descendants, and crowding due to division and growth in the very numerous " killed " cells, might both contribute. For present purposes it is important only that the return to a " normal " mutation rate as indicated by the spray technique is an unreliable indication of the time required for expression of induced mutations.

" Zero-point " mutations

The mutant colonies which arise when irradiated suspensions of *E. coli* B/r are exposed to phage T1 without preliminary incubation have been termed

" zero-point " mutations (DEMEREC and LATARJET 1946), and they could be interpreted as due either to early expression in the occasional induced mutant, or to delayed selection (see LEDERBERG 1949).

The contention that phage selection is grossly retarded in irradiated bacteria is supported by the following properties of these " zero-point " mutations: (1) " zero-point " mutations to phage resistance are much more numerous than the corresponding " zero-point " mutations to streptomycin resistance, despite the evidence that phenotypic expression is slower in the case of phage resistance; (2) with increasing dose an increasing proportion of the mutations to phage resistance are " zero-points " (DEMEREC and LATARJET); (3) with high doses of ultraviolet the number of " zero-points " actually exceeds the number of induced mutations found in single colony isolation studies (cf. DEMEREC and LATARJET figure 1, with table 2 of this paper).

TABLE 5

The absence of demonstrable "zero-point" mutations from low doses of ultraviolet. (Dose = 250 ergs per sq. mm; survival = 44 percent; total mutations to phage T1 resistance = approximately 5000 per 10^8 survivors.)

Replicate samplings	Not irradiated			Irradiated			Estimated "zero-point" mutations (per 10^8)
	Bact. per ml ($\times 10^8$)	Mut. per 0.1 ml	Mut. per 10^8	Bact. per ml ($\times 10^8$)	Mut. per 0.1 ml	Mut. per 10^8	
1	68.3	92	13.5	25.6	39	15.0	+1.5
2	63.8	94	14.7	27.5	35	12.7	−2.0
3	71.9	113	15.8	24.0	40	16.7	+0.9
4	62.1	107	17.2	26.0	38	14.6	−2.6
5	62.5	105	16.7	34.4	49	14.2	−2.5
6	64.7	92	14.2	29.5	42	14.2	0.0
7	58.4	107	18.5	36.4	38	11.5	−7.0
8	53.1	99	18.7	22.3	44	20.0	+1.3
9	70.2	90	12.9	27.6	46	16.5	+3.6
10	66.2	91	13.8	27.7	43	15.5	+2.7
						Average	−0.41

In our experiments low doses of ultraviolet (250 ergs per mm^2, 44 percent survival) sometimes resulted in no " zero-point " mutations (see table 5), although the total number of mutations to phage resistance was high.

Thus, there is further evidence that irradiation reduces the capacity of the bacterium for rapid infection and lysis by phage, and that the heavier the dose the more prolonged the effect.

Interpretation

The difference between the present interpretation of the overall delay and that advanced by WITKIN should perhaps be emphasized at this point. WITKIN postulates as a major contributing factor, a delayed phenotypic expression which differs from that associated with spontaneous mutation (cf. NEWCOMBE 1948) in that the delay is much greater and expression occurs in only a small part of the genetically resistant clone (WITKIN 1951b, p. 367). This view was

adopted to account for the finding of a high proportion of whole-colony lactose mutants in her selection experiments, together with the dependence of this proportion on the time of application of the phage. However, the present data from similar selection experiments fails to show either property, and in addition there is evidence of a selection artifact which under certain conditions causes large numbers of resistant colonies of spontaneous origin to arise where only induced mutants have been expected. This artifact is believed by us to contribute a major part to the apparent long overall delay as estimated using the spray technique, and it might also have produced those features of WITKIN's data which we have been unable to duplicate. This interpretation relieves us of having to assume that induced and spontaneous mutant genes differ so widely in their manner of achieving phenotypic expression.

THE OVERALL DELAY IN APPEARANCE OF INDUCED MUTATIONS TO STREPTOMYCIN RESISTANCE

If, as we have concluded, peculiarities in the phage-host relationship are responsible for both the "zero-point" mutations and the apparent excessive time for return to a normal "mutation rate" following irradiation, it was thought that these phenomena might be absent in the case of other induced changes where a different type of selective agent was used. The mutations to full streptomycin resistance suggested themselves for parallel study because streptomycin could be applied in a wide range of concentrations without inhibiting the mutants. As mentioned earlier, "zero-point" mutations were found to be extremely rare (selecting with 1,000 and 10,000 units of streptomycin per ml).

In our delayed selection experiments about half of the induced mutations were expressed with three hours preliminary incubation, and the remainder by the end of four hours. These times corresponded approximately to population increases of $2^{0.5}$ and 2^{3} fold (see table 6, and NEWCOMBE 1952). Since

TABLE 6

Time required for the expression of ultraviolet induced mutations to streptomycin resistance (and dependence). (Survival = 40 percent.)

Preliminary incubation (hours)	Estimated population increase	Mutant[1] colonies (per 10^8 survivors)
0	2^0	0
1	2^0	25
2	$2^{0.2}$	134
3	$2^{0.5}$	302
4	2^3	857
5	2^6	837
6	2^9	770

[1] 3×10^7 and 3×10^8 bacteria were plated for each incubation period; the above values are calculated from plates having between 50 and 1000 colonies. Unirradiated populations of similar size do not result in more than three or four colonies per plate even with long preliminary incubations.

full expression could have occurred before the end of the four hours the population increase associated with it was somewhere between 2^1 and 2^3 fold.

There is no appreciable discrepancy here between the observed and expected overall delays. As phenotypic delay is virtually absent (indicated by the combined evidence of NEWCOMBE and HAWIRKO 1949 and LEDERBERG 1951) the expected overall delay must be the sum of the mutation, segregation and irregularity delays. The first two combined probably do not exceed two divisions (and might be one division); the third has not been measured but would seem in this case to be somewhere between zero and two divisions.

It is of course necessary to consider whether streptomycin accurately tests the genotype or the phenotype at the time of application, since the apparent absence of appreciable phenotypic delay might be due to the genotypic or physiological changes leading to resistance being completed in the presence of the streptomycin. However, the rarity of "zero-point" mutations suggests that any such effect might be negligible.

Thus the discrepancy observed in the case of mutations to phage resistance does not occur in a similar system using a chemical, instead of a living, selective agent.

THE COPY-ERROR CONCEPT IN INDUCED MUTATIONS

It should be emphasized that none of the evidence so far obtained is incompatible with the view that induced mutations occur at the time of gene reproduction, there being sometimes one and sometimes two mutant daughter genes. If this concept were correct the early mutations in an irradiated population would be those associated with early gene division and would produce whole-colony and sectored mutants in the same proportions as later mutations.

Two methods are available by which early mutations could perhaps be singled out for study: (1) those to phage resistance might be selected by treating with phage after short preliminary incubations, and (2) those to lactose non-fermentation might be obtained by exposing ultraviolet irradiated material to visible light after limited periods of incubation with a view to terminating the mutagenic action prematurely. In both cases the proportions of whole-colony and sectored lactose mutants would be observed.

If the hypothesis were wrong, and early mutations produced whole-colony mutants, then in the phage selection experiment a short preliminary incubation should favour the lactose sectorials (since the opportunity for sectoring is greatest when all the descendants from an irradiated nucleus survive), and in the photoreversal experiment a short incubation before exposure to light should favour the whole-colony lactose mutants. If, on the other hand, all mutations occur at the time of gene division, the timing of the phage selection and of the exposure to light should have no effect on the ratio of whole to sectored colonies. Both experiments were carried out (see tables 7 and 8).

In the case of the phage selection, preliminary incubations of one and four hours were used. A slight difference was found (see table 7) but this was within the limits of experimental error and, even if real, would indicate only

TABLE 7

The effect of varying the period of preliminary incubation in phage selection experiments upon the proportions of "whole" and "sectored" lactose mutations, (Ultraviolet dose = 400 ergs per mm².)

Preliminary incubation (hours)	Colonies examined	Lactose (whole : sect.)	Mutations	
			Corrected[1] ratio	Percent whole
1	31,205	24 : 21	21 : 15	58
4	33,856	40 : 20	39.5 : 19.0	67

[1] Corrected for lactose mutations in phage resistant cells present at the time of irradiation. 4,840 of the colonies from the one hour incubation and 736 from the four hour incubation were from such cells. The number of lactose mutations in these is assumed to be $19/10^4$, in the ratio of one "whole" to two "sectored" (see table 3).

a small proportion of genuine pre-gene-division mutations. The delayed photoreversal resulted in no excess of whole-colony lactose mutants (see table 8) and thus gave no indication of variations in the timing of induced mutations with respect to gene division.

Neither result entirely rules out the possibility of pre-gene-division and post-gene-division mutations, since it is just possible that phenotypic delay rendered the phage selection experiments too insensitive to reveal small differences in timing, and that the light acted to interrupt the chain of events at a point one or more steps prior to the actual gene change (as for example by preventing the conversion of a photo-sensitive mutagen into a photostable form). However, taken together the two experiments point to a close relationship between the times of gene division and of induced gene mutation.

There are at present two commonly held concepts of gene mutation: (a) as a molecular change caused by mutagenic chemicals within the cells, and (b) as an inaccurate duplication of the gene. The first has recently been expanded to include all gene mutations of whatever origin (see McElroy and Swanson

TABLE 8

The effect of delayed photoreversal on the proportions of "whole" and "sectored" lactose mutations. (Ultraviolet dose = 400 ergs per mm². No phage selection.)

Treatment[1] (ultraviolet, incub. in min., and light)	Colonies examined	Lactose mutations		
		Whole : sect.	Percent whole	Mut. per 10^4
Control	57,480	10 : 28	....	6.6
UV	120,337	61 : 131	32	16.0
UV–0–L	120,860	13 : 46	....	4.9
UV–30–L	45,860	4 : 19	....	5.0
UV–60–L	79,650	15 : 62	20	9.7

[1] Note: Percents survival, using the combined data, are 2.4, 37, 33, and 17, for UV, UV–0–L, etc. (From this it is evident that the lethal and mutagenic effects of ultraviolet start to become stable towards light after similar periods of incubation.)

1951, p. 360) while the second has been considered mainly in relation to spontaneous gene change. The evidence from spontaneous mutation appears to be about equally divided for and against the latter concept, mutation being in some experiments a function of the number of divisions and independent of time (LURIA and DELBRÜCK 1943; ZAMENHOF 1945; NEWCOMBE 1948, table 4), and in others a function of time and independent of division (NOVICK and SZILARD 1950; see also KAUFMANN 1947 for reference dealing with organisms other than bacteria). However, both conclusions appear to be valid under the conditions of the experiments upon which they are based, and the apparent conflict could be resolved by combining the two concepts, the amount of mutagen formed in the cell determining the likelihood of a copy error during gene duplication.

The experiments on induced mutations in bacteria indicate that gene changes take place only after a period of metabolic activity, and at a time corresponding approximately to that of gene division. Thus although neither spontaneous nor induced mutations have as yet been demonstrated to occur during gene duplication, the possibility is not ruled out by any of the evidence from either source, and all of the phenomena associated with mutation could be interpreted on the combined concept of chemical mutagens (either natural, radiation induced, or artificial) acting to produce inaccuracies in gene copying.

If this combined concept is correct two possibilities are opened up. First, information concerning the process of gene reduplication might be derived from the sectoring phenomenon in bacteria and the "fractional" mutations of higher organisms. Second, there is a possibility that some effective post-treatment might yet be found for the radiation-induced changes which lead to genetic damage.

Further, it is known that the syntheses of certain intracellular enzymes (see SWENSON and GIESE 1950, BRANDT et al. 1951, BILLEN and LICHSTEIN 1952) and of desoxyribonucleic acid (KELNER, unpublished) are inhibited by doses of irradiation which, in so far as has been determined, have little effect on the substances themselves. This suggests an analogy with the assumed influence of irradiation on gene synthesis, and although the analogy is at present incomplete, the observed effect being quantitative in the one case and qualitative in the other, there may well be a fundamental similarity, both types of synthesis being partially inhibited and in addition yielding occasional variant end-products. Thus it is conceivable that irradiation (and presumably mutagens in general) affect in a like manner a whole group of fundamentally similar syntheses of which gene reduplication is only one.

SUMMARY

The bulk of induced mutations to phage T1 resistance in *Escherichia coli* become apparent only after appreciable growth of the irradiated suspensions. This could be due to delays associated with: (1) gene mutation, (2) nuclear segregation, (3) phenotypic expression, and (4) irregularity in onset of divi-

sion. These delays are estimated by various means to be equal to population increases of 2^1- to 2^2- (for 1 and 2 combined), 2^2- to 2^3-, and 2^0- to 2^2-fold respectively, making an overall delay of between 2^3- and 2^7-fold.

The discrepancy between this and the much higher estimates (2^{13}-fold) obtained directly by the phage spray technique could be due to a phage selection artifact in the latter experiments. Our evidence supports this view; and in addition, no similar discrepancy was found in the case of another mutation (to streptomycin resistance) where a different type of selective agent was used.

Special attention has been paid to the timing of induced gene changes, and attempts to show a relationship between the earliness of a mutation and the likelihood of sectoring in the resulting colony, have failed. This suggests that gene changes might be limited to the period of duplication, affecting sometimes one and sometimes both daughter genes.

The theory that spontaneous and radiation-induced gene changes both occur in response to mutagenic substances formed in the cell is extended to include the possibility that these substances interfere with a whole group of essentially similar syntheses of which gene reproduction is one.

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IMMUNOGENETIC STUDIES OF DROSOPHILA MELANOGASTER. III. FURTHER EVIDENCE OF GENIC INTERACTION IN THE DETERMINATION OF ANTIGENIC SPECIFICITY[1]

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A PREVIOUS paper described the results of the antigenic analysis of a series of co-isogenic stocks consisting of a reference stock (" Isogenic "), two single-mutant stocks derived from it (ruby and vermilion), and a double-mutant stock (ruby-vermilion) derived from the single mutants (Fox 1949b). It was found that Isogenic possessed an antigenic fraction, subsequently called Antigen 1, not possessed by the mutant stocks. Vermilion and ruby-vermilion were found to possess a different antigenic component (Antigen 2) not present in Isogenic and ruby. Ruby could not be demonstrated to possess a specific antigenic fraction. Assuming that the mutant stocks differed from Isogenic only with respect to the vermilion and ruby loci, it was concluded the Antigen 1 was the interaction product of the wild alleles of these loci.

The present investigation consists of two parts. The first part comprises a test of the above assumption and yields evidence supporting its validity. The second part consists of a test which indicates that one or more loci in addition to ruby and vermilion participate in the production of Antigen 1. An addendum describes the partial chemical isolation and characterization of Antigen 1.

MATERIALS AND METHODS

Genetic. The four co-isogenic stocks used in the previous investigation were of the following genotypes:

Isogenic	*Payne Dfd ca/1(3)F*
Vermilion	*v; Payne Dfd ca/1(3)F*
Ruby	*rb; Payne Dfd ca/1(3)F*
Ruby-Vermilion	*rb v; Payne Dfd ca/1(3)F.*

Isogenic was derived by means of the familiar " marker, crossover-inhibitor " method, so that the X chromosome, the second chromosome and each of the third chromosomes traced to a single source. The two mutations were X-ray-induced, and the single mutant stocks were derived by replacing rayed autosomes with untreated chromosomes from Isogenic. The double mutant stock was derived from a crossover in an F_1 female produced in a cross between the single-mutants. The two mutants are listed respectively as rb^{48a} and v^{48a} in Drosophila Information Service, No. 22, 1948.

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The results of the investigation indicated that the possible heterogeneity of fourth chromosomes in the stocks was not responsible for the observed differences. However, since a dose of 3000 r was used to induce the mutations, it was not possible to rule out the occurrence of iso-allelic mutations of sex-linked loci other than ruby and vermilion. Consequently the possibility that the observed results could be attributed to mutation at loci other than these two remained to be considered.

In this investigation these four stocks have been utilized in the derivation of two new series of stocks, the details of which are to be found below.

Immunological. The immunological technics used in this investigation were identical with those previously reported (Fox 1949a, 1949b). In general terms, the analysis of each antiserum is performed in several steps. Initially, the optimal-proportion point exhibited by each antiserum with the antigens of each of the several stocks is determined. These tests are performed in a series of tubes containing like amounts of antiserum but decreasing amounts of antigen. After the optimal-proportion determination the tubes are incubated for a period of time sufficient to allow for completion of the antigen-antibody reaction, centrifuged, and the supernates used as absorbed sera in precipitin-ring tests with each of the antigens. In these latter tests, the antigens are dissolved in gelatin in concentrations proportional to their optimal-proportion ratios with the serum being tested. The results of the precipitin-ring tests indicate the existence of antigenic fractions specific to given stocks or held in common among two or more of the stocks.

One point of difference between the results of this investigation and those previously reported, having an influence on the performance of the tests, should be noted. In the previous work, the optimal-proportion point exhibited by a serum with its homologous antigen was frequently markedly lower than with heterologous antigens. As a result, the concentration of homologous antigen used in the precipitin-ring tests was correspondingly higher than the concentrations of heterologous antigens. In the present work, such differences in optimal-proportion points were not encountered, and all antigens were used in the same concentration in the precipitin-ring tests. As will be seen below, this difference does not influence the results of the tests or the conclusions derived therefrom, and independent tests have demonstrated that antigen concentration is not critical in the precipitin-ring tests.

It may thus be seen that the optimal-proportion technic is used primarily as a method of locating appropriate equivalence zones so as to assure complete absorption with a given antigenic preparation. In has resulted in economies of labor and material and has proved effective where dilutions of antisera are impossible because of low titer. All conclusions, however, are based on the results of the precipitin-ring tests of antisera absorbed in this manner.

EVIDENCE SUPPORTING THE CONCLUSION OF INTERACTION BETWEEN rb^+ AND v^+

Stocks. The major source of doubt confronting the conclusions previously reached was the possibility that undetected mutation at X-chromosomal loci

other than *rb* and *v* was responsible for the antigenic differences demonstrated. This possibility has been tested by attempting to separate such mutations from *rb* and *v* by means of crossing over. The series of stocks derived in this attempt will henceforth be called series 1.

In preparation for this derivation, a Muller-5 (sc^{8I} *B In-S* w^a sc^8) stock with autosomes derived from Isogenic was constructed as follows:

M-5/M-5; Cy/Pm, ds^{33k}*; H/In(3R)Mo, Sb sr*
$\times$ *Payne Dfd ca/1(3)F* (Isogenic)

to yield females of the following genotype:

M-5/+; Cy/+; In(3R)Mo, Sb sr/1(3)F.

Such females were then mated with Isogenic males yielding the following males and females:

M-5/Y; +/+; Payne Dfd ca/1(3)F
M-5/+; +/+; Payne Dfd ca/1(3)F.

These were mated inter se giving:

M-5/Y; +/+; Payne Dfd ca/1(3)F
M-5/+; +/+; Payne Dfd ca/1(3)F,

which were then used to derive the desired stock.

The actual derivation of series 1 then proceeded in the following fashion. A single ruby-vermilion male was mated with a single Isogenic female to yield *rb v/rb*$^+$ *v*$^+$*; Payne Dfd ca/1(3)F* daughters. Crossing over between *rb* and *v* in a single such female yielded an X chromosome carrying only *v* and a second X carrying only *rb*. These were isolated without further crossing over by means of a series of crosses beginning with this heterozygous female:

rb v/rb$^+$ *v*$^+$*; Payne Dfd ca/1(3)F* $\times$ *Payne Dfd ca/1(3)F* (Isogenic).

From among the progeny of this female a single *rb* male and a single *v* male were chosen for matings with females from the co-isogenic Muller-5 stock derived above:

rb v$^+$*/Y; Payne Dfd ca/1(3)F* $\times$ *M-5/M-5; Payne Dfd ca/1(3)F*
and
rb$^+$ *v/Y; Payne Dfd ca/1(3)F* $\times$ *M-5/M-5; Payne Dfd ca/1(3)F.*

The first of these matings yielded

rb v$^+$*/M-5; Payne Dfd ca/1(3)F*

females, which were then mated with their father:

rb v$^+$*/M-5; Payne Dfd ca/1(3)F* $\times$ *rb v*$^+$*/Y; Payne Dfd ca/1(3)F*

to yield

rb v$^+$*/Y; Payne Dfd ca/1(3)F*
and
rb v$^+$*/rb v*$^+$*; Payne Dfd ca/1(3)F,*

which when mated established a new ruby stock (ruby 1). The second of the above matings by means of a similar series of crosses yielded a new vermilion stock (vermilion 1).

New type and ruby-vermilion stocks were derived from a mating between a single ruby 1 female and a single vermilion 1 male which yielded heterozygous daughters: *rb*$^+$ *v*/*rb v*$^+$; *Payne Dfd ca/1(3)F*. Crossing over in a single such female yielded a reconstituted *rb v* X chromosome and a reconstituted wild X. These were isolated without further crossing over by means of series of matings similar to those used in the derivation of ruby 1 and vermilion 1, and were incorporated in the new ruby-vermilion 1 and type 1 stocks.

It may be seen that while the autosomes in each of these new stocks may be assumed to be the same as those in the original stocks (including *Payne Dfd ca/1(3)F*), the X chromosomes have been altered by crossing over between *rb* and *v*. In the case of ruby-vermilion 1 and type 1 two occasions of such crossing over have occurred. If the antigenic differences among the original stocks were due to undetected mutations at loci other than *rb* and *v*, it may be hoped that these have been separated from *rb* and *v* during the course of this experiment.

Results. Antisera to each of the stocks in series 1 have been analyzed. Since the analysis of each serum includes four optimal-proportion determinations and tests of the supernates from each optimal-proportion determination with antigens of each of the four stocks, the full results cannot be given here. Instead, we have chosen to present in detail the results of one test which may serve as a typical example, and have summarized the remaining results in a brief form.

Table 1 contains the results of the analysis of a single anti-type 1 serum which discloses the antigenic structure of type 1 as compared with vermilion 1. Part a of the table contains the protocol and results of the optimal-proportion test of this serum with vermilion 1 antigens. The optimal-proportion is seen to equal 0.8, a very low value. Such low optimal-proportion points have been frequently observed in this work, and would seem to indicate that the active components of the antigenic preparations are present in minute amounts. The optimal-proportion points exhibited by this serum with the antigens of the other stocks in series 1 were approximately equal to that obtained in this test.

After suitable incubation, the supernates from the optimal-proportion determination were used in precipitin-ring tests with the antigens of each of the four stocks (dissolved at a concentration of 1 : 2 in 5% gelatin). The results are contained in part b of the table. Antigenic, antiserum and normal controls are omitted from the table but were all negative. It may be seen that quantities of vermilion 1 antigens larger than 0.25 ml completely remove from a 0.4 ml sample of a 1 : 2 dilution of the serum all antibodies capable of reacting with vermilion 1 antigens (tubes 1, 2 and 3). The absorption is equally or more effective with respect to ruby 1 and ruby-vermilion 1, but it is apparent that vermilion 1 is incapable of exhausting the serum of antibodies which react with type 1. This observation serves, then, to identify an antigenic component

(simple or complex) possessed by type 1 but not by the mutant members of series 1.

The results contained in table 1 of this publication are sensibly identical with table 4 of our previous publication (Fox 1949b) which contained the results of the analysis of the original Isogenic stock with respect to the original vermilion stock. In at least this respect, the derivation of type 1 appears not to have resulted in a change of antigenic structure. Three additional anti-type 1 sera have given similar results. (This is not to be construed as meaning that all anti-type 1 sera exhibit this specificity. In our experience only about

TABLE 1

Analysis of type 1 with respect to vermilion 1.

a. Exact Optimal-Proportion Test

Serum: S-50, anti-type 1; dilution, 1:2. Antigen: F-3d-3, vermilion 1. Optimal proportion[1]: 0.8. Incubation temperature: 37.5°C.

Tube	1	2	3	4	5	6	7	8
Saline (ml)	....	0.1	0.15	0.2	0.23	0.25	0.27	0.29
Antigen (ml)	0.4	0.3	0.25	0.2	0.17	0.15	0.13	0.11
Antigen dilution	1:1	1:1.3	1:1.6	1:2	1:2.4	1:2.7	1:3.1	1:3.6
Antiserum (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Time to pp't. (min)	20	19.5	18	23	>23	>23	>23	>23

[1] Optimal proportion $= \frac{\text{Dilution of antigen}}{\text{Dilution of antiserum}} = \frac{1.6}{2}$.

b. Precipitin-Ring Test

Above tubes incubated at 37.5°C for 2 hours followed by 6°C for 24 hours, centrifuged, and supernates used in following:

Antigen	Tube							
	1	2	3	4	5	6	7	8
F-1c-2, type 1 (1:2 in gel.)	++	++	++	++	++	++	++	+++
F-2c-2, ruby 1 (1:2 in gel.)	–	–	–	–	–	tr	tr	+
F-3d-3, vermilion 1 (1:2 in gel.)	–	–	–	tr	tr	+	+	++
F-4b-4, ruby-vermilion 1 (1:2 in gel.)	–	–	–	–	tr	tr	+	++

one out of five antisera is specific. This is an experience duplicated in most immunological work (IRWIN 1949) and appears to result from a difference in responsiveness of rabbits to antigenic stimuli.)

In table 2 are summarized in a qualitative fashion the results of the rest of the analysis of type 1, along with the results of the analysis of vermilion 1 (two specific sera), ruby 1 (eight antisera), and ruby-vermilion 1 (two specific sera). The table discloses the existence of two specific fractions. The completed analysis of anti-type 1 serum serves further to identify the antigenic component mentioned above; namely, a component specific to type 1 but not possessed by vermilion 1, ruby 1, or ruby-vermilion 1. In addition, analysis of

vermilion 1 and ruby-vermilion 1 anti-serum yields evidence of a second component, possessed by these two stocks but not present in type 1 or ruby 1.

Comparison of this table with table 6 in Fox (1949b) reveals that the antigenic structure of the series 1 stocks is identical to that of the original stocks. In particular, the type 1-specific component exhibits a distribution identical with that of Antigen 1, and the distribution of the component possessed by vermilion 1 and ruby-vermilion 1 parallels that of Antigen 2. These parallelisms argue, therefore, for an identification of the two specific fractions of series 1 with Antigens 1 and 2 of the original series of stocks.

Further evidence for such an identification is contained in table 3. When an anti-Isogenic serum was absorbed by vermilion 1 it no longer reacted with vermilion 1, ruby 1 or ruby-vermilion 1, but reacted with equal intensity with

TABLE 2

Summary of analysis of series 1.

Antiserum	Absorbed by	Tested with			
		Type 1	Vermilion	Ruby 1	Ruby-vermilion 1
Anti-type 1	Type 1	–	–	–	–
	Vermilion 1	+	–	–	–
	Ruby 1	+	–	–	–
	Ruby-vermilion 1	+	–	–	–
Anti-vermilion 1	Type 1	–	+	–	+
	Vermilion 1	–	–	–	–
	Ruby 1	–	+	–	+
	Ruby-vermilion 1	–	–	–	–
Anti-ruby 1	Type 1	–	–	–	–
	Vermilion 1	–	–	–	–
	Ruby 1	–	–	–	–
	Ruby-vermilion 1	–	–	–	–
Anti-ruby-vermilion 1	Type 1	–	+	–	+
	Vermilion 1	–	–	–	–
	Ruby 1	–	+	–	+
	Ruby-vermilion 1	–	–	–	–

Isogenic and type 1. When an anti-vermilion (original series) serum was absorbed with type 1 antigens, it no longer reacted with type 1 or ruby 1, but reacted with equal intensity with vermilion (original series), vermilion 1 and ruby-vermilion 1. There seems to be little doubt that the type 1-specific component is Antigen 1 and that the component possessed by vermilion 1 and ruby-vermilion 1 is Antigen 2. (More extensive tests were prevented by the unfortunate loss of the original series of stocks to the combined attack of summer heat and mold.)

The failure of anti-ruby and anti-ruby 1 sera to demonstrate a ruby-specific component deserves further comment. As has been previously pointed out (Fox 1949b), such failure is not conclusive evidence that ruby and ruby 1 do not possess a specific component. However, a total of ten anti-ruby sera have been analyzed, two against the original ruby and eight against ruby 1, without

positive results. If a ruby-specific component exists is must certainly not be one of high antigenicity.

Discussion. The test described in the preceding pages is essentially one of the inseparability of Antigens 1 and 2 from change at the ruby and vermilion loci. Opportunity for recombination between these loci and the observed antigenic properties has been provided. No recombination has occurred.

It is fully realized, of course, that inseparability can be conclusively demonstrated only by means of repeated tests such as have been reported here. The

TABLE 3

Tests for identity of antigens.

a. Antigen 1

Serum: S-13, anti-isogenic. Absorbed by F-3d-3, vermilion 1. Tested with following antigens in precipitin-ring tests.

Antigen (in gel. 5%)	Antigen dilution	1:2	1:4	1:8	1:16	1:32	1:64
Isogenic (D-1-5)		++++	++++	+++	++	++	+
Type 1 (F-1c-2)		++++	++++	+++	++	++	+
Vermilion 1 (F-3d-3)		–	–	–	–	–	–
Ruby 1 (F-2c-2)		–	–	–	–	–	–
Ruby-vermilion 1 (F-4b-4)		–	–	–	–	–	–

b. Antigen 2

Serum: S-15, anti-vermilion. Absorbed by F-1c-3, type 1. Tested with following antigens in precipitin-ring tests.

Antigen (in gel. 5%)	Antigen dilution	1:2	1:4	1:8	1:16	1:32	1:64
Vermilion (D-3-3)		++++	++++	+++	+++	++	++
Vermilion 1 (F-3d-3)		++++	++++	++++	+++	+++	++
Ruby-vermilion 1 (F-4b-4)		++++	++++	+++	++	++	++
Type 1 (F-1c-2)		–	–	–	–	–	–
Ruby 1 (F-2c-2)		–	–	–	–	–	–

labor involved in the analysis of additional series of stocks is, however, prohibitive and, when the total picture is viewed, seems unprofitable to pursue.

It should be recalled that the method of derivation of the original series of stocks was such as to reduce to a minimum the probability that genetic differences other than those at the ruby and vermilion loci existed among the stocks. The method of the derivation of co-isogenic stocks by means of the induction of mutations on an isogenic background seems preferable even to the method of inbreeding with forced heterozygosis (SCHWAB 1940; DOBZHANSKY and

HOLTZ 1943). In our previous discussion of the methods used to derive the original series of stocks (FOX 1949b) we commented that two possible sources of uncontrolled genetic variability among the stocks were 1) secondary effects of the X-ray treatment, and 2) lack of control of chromosome 4. The former possibility is tested in the present work. With respect to the latter possibility we stated that in the original series of stocks, " due to the method of their derivation, the mutant stocks would be expected to possess, among others, a sample of fourth chromosomes derived from the Isogenic stock. This being the case, Isogenic antigens produced by loci on the fourth chromosome might be expected to occur in the mutant stocks as well. It will be recalled that Isogenic was demonstrated to possess an antigenic fraction *not* possessed by the mutant stocks. This circumstance, along with the small size of the fourth chromosome, would seem to reduce, although not eliminate, the possibility that the antigenic differences between the stocks are due to genetic variability of the fourth chromosome." This comment is equally pertinent for the present work.

Another comment which should be made about the methods used in both the previous and present work is that the presence of inversions frequently causes crossing over to occur in regions where it would otherwise be negligible (STURTEVANT 1919; MORGAN, BRIDGES and SCHULTZ 1930; SCHULTZ 1951). This could be an additional source of uncontrolled genetic variability, but the results of these investigations would seem to indicate that any such variability has not had antigenic effect. The opportunity for the introduction of variability from this source has occurred during the derivation of each of the four original stocks and each of the four stocks of series 1. But if such variability has been introduced it would be expected to differ from stock to stock, and if accompanied by antigenic effect would not have resulted in the consistent pattern of antigenic distribution observed in both series of stocks.

The methods therefore seem reliable, and added to these assurances are the results of the present tests. There seems little cogent doubt that the antigenic differences demonstrated in this work are attributable to the differences at the ruby and vermilion loci. It may be concluded, therefore, that Antigen 1 is the interaction product of v^+ and rb^+, and that Antigen 2 is associated with mutation at the vermilion locus.

This being the case, an additional question may now be formulated. While interaction between rb^+ and v^+ may be considered to have been demonstrated, there remains the possibility that other loci are also involved in this interaction. Since all of the stocks analyzed have the same residual genotype they are not useful for an examination of this question. The second part of this paper is devoted to an analysis of the antigenic effects of changes in the residual genotype.

EVIDENCE INDICATING THE PARTICIPATION OF ADDITIONAL LOCI IN THE PRODUCTION OF ANTIGEN 1

Stocks. A series of stocks (series 3) possessing a different residual genotype was derived by introducing autosomes from an unrelated, non-isogenic source into each of the four stocks of series 1. The derivation of the ruby 3

stock may be taken as an example. Ruby 1 males were mated with Muller-5 (sc^{8I} *B In-S* w^a sc^8) females: *rb/Y; Payne Dfd ca/1(3)F* × *M-5/M-5*. Bar Deformed female offspring were crossed with Muller-5 males: *M-5/rb; Payne Dfd ca/+* × *M-5/Y*. Heterozygous Bar females were mated with ruby brothers: *M-5/rb; +/+* × *rb/Y; +/+*. Ruby males and females from this cross were mated inter se, thus establishing the ruby 3 stock. Similar matings were used in the derivation of type 3, vermilion 3 and ruby-vermilion 3.

The X chromosome in each of these stocks is derived unchanged from the corresponding series 1 stock. The third chromosomes of the series 1 stocks have been replaced by Muller-5 third chromosomes, and each stock probably possesses a sample of Muller-5 second and fourth chromosomes. It should be

TABLE 4

Summary of analysis of series 3.

Antiserum	Absorbed by	Tested with			
		Type 3	Vermilion 3	Ruby 3	Ruby-vermilion 3
Anti-type 3	Type 3	–	–	–	–
	Vermilion 3	–	–	–	–
	Ruby 3	–	–	–	–
	Ruby-vermilion 3	–	–	–	–
Anti-vermilion 3	Type 3	–	+	–	+
	Vermilion 3	–	–	–	–
	Ruby 3	–	+	–	+
	Ruby-vermilion 3	–	–	–	–
Anti-ruby 3	Type 3	–	–	–	–
	Vermilion 3	–	–	–	–
	Ruby 3	–	–	–	–
	Ruby-vermilion 3	–	–	–	–
Anti-ruby-vermilion 3	Type 3	–	+	–	+
	Vermilion 3	–	–	–	–
	Ruby 3	–	+	–	+
	Ruby-vermilion 3	–	–	–	–

noted that series 1 was found to possess Antigen 1 and Antigen 2 both before and after the derivation of series 3.

Analysis of series 3. As in the case of series 1, the results of the complete analysis of series 3 are too lengthy for full presentation here. The most important result is, however, characterized by the details of the analysis of anti-type 3 serum with respect to vermilion 3. In contrast with the result of comparable tests of series 1, it is found that vermilion 3 antigen is capable of completely exhausting, by absorption, the anti-type 3 serum of antibodies capable of reaction with type 3. In general, the serum reacts no more strongly with type 3 than with the mutant antigens. From these observations it is concluded that type 3 does not possess an antigenic component not also possessed by vermilion 3. Twelve anti-type 3 sera have been analyzed and all have given the same result.

The complete analysis of type 3, the qualitative summary of which is contained in table 4, reveals that anti-type 3 serum reacts similarly with the antigens of all four stocks in all tests. Similar but less extensive tests of type 3 antisera with antigens of the series 1 stocks have given similar results. It appears, therefore, that type 3 does not possess Antigen 1, and this has been confirmed by means of cross-reaction tests, similar to those contained in table 3, with type 1 antisera capable of reacting with Antigen 1.

Table 4 also contains the results of the analysis of vermilion 3 (two specific sera), ruby 3 (four antisera), and ruby-vermilion 3 (two specific sera). The antigenic pattern exhibited by these stocks is identical with that exhibited by the corresponding original and series 1 stocks. While ruby 3 cannot be demonstrated to possess a specific fraction, vermilion 3 and ruby-vermilion 3 share an antigenic component not present in type 3 or ruby 3. This latter component exhibits a distribution typical of Antigen 2. The results contained in table 5 demonstrate that vermilion 3 and vermilion 1 react in a similar fashion

TABLE 5

Test for Antigen 2.

Serum: S-31, anti-vermilion 1. Absorbed by F-1-1, type 1. Tested with following antigens in precipitin ring test.

Antigen (in gel, 5%)	Antigen dilution	1:2	1:4	1:8	1:16	1:32	1:64
Vermilion 1 (F-3d-1)		++++	++++	+++	++	+	+
Vermilion 3 (G-3c-1)		++++	++++	+++	++	++	+
Type 1 (F-1-1)		–	–	–	–	–	–

with anti-vermilion 1 serum, thus indicating that the vermilion 3, ruby-vermilion 3 specific component is undoubtedly Antigen 2.

Disappearance of Antigen 1 from type 1. The derivation of series 1 was completed in January, 1949 and the stocks were maintained thereafter by means of mass cultures. Type 1 repeatedly gave positive tests for the presence of Antigen 1 through all of 1949 and 1950, but difficulty was encountered in tests performed during early part of 1951 until finally no indication of the presence of Antigen 1 in the stock could be obtained. Repeated tests performed since that time have indicated Antigen 1 has permanently disappeared from the stock.

Examination of our records makes it possible to fix the time of this disappearance in February of 1951. Antigens prepared from the stock since that date have failed to react with antisera previously demonstrated to contain antibodies for Antigen 1, and antisera against antigens prepared since that date fail to react with preparations known to contain Antigen 1. The records also seem to exhibit a gradual decrease of Antigen 1 in the stock leading up to its final disappearance.

Since, after the loss of the original Isogenic stock, type 1 was the only stock available to us containing Antigen 1, it has been impossible to subject this disappearance to genetic analysis. The above observations, however, make certain suggestions highly likely, and these will be discussed below.

Absence of Antigen 1 from Oregon R. A series of co-isogenic stocks with an Oregon R background has been made available to us through the courtesy of DR. JACK SCHULTZ, and has been subjected to preliminary analysis, the results of which will be published elsewhere when complete. The analysis has gone far enough, however, to indicate that the wild stock in the series (designated by SCHULTZ as Oregon R, series I) does not possess Antigen 1. Since the genetic background of Oregon R is different from that in our stocks, this observation has pertinence for the present discussion.

Discussion. The conclusion to be derived from the results of the analysis of series 3 seems relatively clear. Since alteration of the residual genotype has resulted in the absence of Antigen 1 from type 3 despite the presence of rb^+ and v^+, at least one additional, autosomal locus must be involved in the interaction producing Antigen 1. More than one additional locus may be involved, of course, and the absence of Antigen 1 from the Oregon R stock would indeed seem to lend weight to this latter supposition.

With this conclusion in mind, the disappearance of Antigen 1 from type 1 becomes easily explicable. This stock was maintained by mass culture, which means that at the time of each transfer (approximately every fourth week) an unselected sample of about 50 individuals served as parents of the subsequent generations. Such a breeding system is ideally suited for random fluctuations in gene frequencies of the sort discussed by WRIGHT (1948). If, then, Antigen 1 is the product of interaction of a number of loci, isoallelic mutation of at least one of these could readily occur and the frequency of the new isoallele could gradually rise in the stock due to sampling fluctuations even in the absence of selective advantage. Accompanying this rise in gene frequency would occur a fall in the amount of Antigen 1 in the stock, culminating in its complete disappearance. Thus, the disappearance of Antigen 1 is not only explicable if interaction of a number of loci is involved, but actually serves to support that conclusion.

Antigen 2, on the other hand, is still present in vermilion 3 and ruby-vermilion 3 despite the change in residual genotype. In other words, no interaction has been demonstrated for this antigen which seems, therefore, to be associated only with mutation at the vermilion locus. It is possible, of course, that other loci are concerned with Antigen 2 but have not been detected because they have not been changed by the substitutions of residual genotype made in the derivation of series 3. On the other hand, if this is the case the number of such loci is certainly not as large as for Antigen 1. Such considerations are hypothetical, of course, but they serve the purpose of demonstrating that while interaction can be shown in certain cases, the absence of interaction is difficult to establish.

GENERAL DISCUSSION

This series of investigations was initiated for the purpose of examining the validity of the one gene-one antigen hypothesis in material capable of critical genetic manipulation. Evidence supporting that hypothesis has come largely from immunogenetic studies of vertebrate erythrocytic antigens (IRWIN and COLE 1936; HALDANE 1938; IRWIN 1951), where interaction of genes at different loci in the determination of antigenic specificity has appeared to be absent or extremely rare. As an extension of the hypothesis it has been postulated that the antigens of red blood cells are the direct or primary products of their causative genes.

The results of the present investigations have from the start been in apparent contradiction to the one-to-one hypothesis. We have previously suggested that there may exist a fundamental difference in the genetic determination of the specificity of erythrocytic antigens as compared with the determination of the specificity of proteins (Fox 1949b). On the basis of subsidiary considerations, we proposed the possibility that while the one-to-one relationship may hold for cellular antigens (which are not proteins), genic interaction might be the rule in the production of proteins. Subsequent examination of newer evidence has, however, led us to the conclusion that no fundamental difference exists in the genetic determination of the specificity of cellular antigens and proteins. The reasons for this conclusion will be discussed in a separate publication.

It should be pointed out, however, that STORMONT, OWEN, and IRWIN (1951) have correctly indicated that there are two consequences of the one-to-one hypothesis that bear examination. In the first place, the hypothesis requires that no single antigenic specificity be the product of more than one gene. Conversely, no one gene should be responsible for more than one antigenic specificity. The present case is clearly an exception to the first aspect of the hypothesis.

In a previous publication (Fox 1949b) we suggested a number of alternative hypotheses concerned with the chain of events leading to the production of Antigens 1 and 2 and consistent with the conclusions reached concerning their genetic control. The results of the present investigation, demonstrating that one or more loci in the residual genotype are concerned with the production of Antigen 1, clearly rule out all but one of the five hypotheses presented there and this one is to be found in figure 1. According to the suggestion contained therein, *rb* is an amorph; rb^+ utilizes a non-antigenic substrate which it converts into a non-specific precursor; v^+ converts a non-antigenic substrate into a second non-specific precursor; *v* utilizes the same substrate as its wild allele, but converts it into Antigen 2; the two non-specific precursors, when produced, are coupled by the action of one or more loci of the residual genotype, during the course of which process specificity is conferred on the resulting protein, i.e. Antigen 1.

This diagram is, of course, a formal one and should not be taken as literally representing the processes involved in the production of the antigens. A number of other diagrams fitting the data equally well might be proposed. In this connection, a point of interest concerning the vermilion locus arises. In figure 1, v is represented as a neomorph, competing with its wild allele for a common substrate but converting it into a different product. It is equally possible, however, that v is an amorph, in which case minor modification of the diagram would be necessary to render it consistent with the data (the substrate for v^+ would become Antigen 2 rather than a non-antigenic substance).

GREEN (personal communication) has kindly given us permission to mention a further discovery which is pertinent in connection with vermilion. By special means he has succeeded in demonstrating crossing over between v^1 and v^{36f}, occurring with a frequency of the order of 1/25,000 and indicating that different vermilion mutants are pseudoalleles rather than alleles and that the vermilion "locus" is in reality two closely linked loci. Further, in one trial v^{48a}/v^1 females yielded no cross-over progeny among 60,000 offspring. This

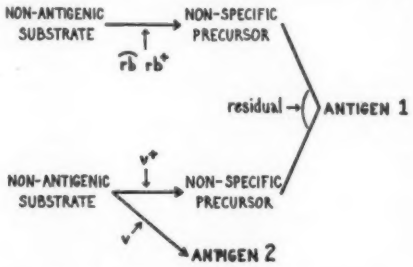

FIGURE 1.—Hypothesis accounting for observed gene-antigen relationships.

would suggest that v^{48a} is allelic with v^1 rather than with v^{36f}, but GREEN strongly suggests, among other possibilities, that v^{48a} is actually a double mutant, allelic to both v^1 and v^{36f}. The suggestion is based on the observation that the v "locus" is in all probability a very clear doublet in the salivary chromosomes, and on the plausibility of the supposition that X-ray-induced mutations (like v^{48a}) could involve both halves of the doublet and therefore both loci. In any event, an investigation of the antigenic effects of different vermilion mutants is clearly indicated, and is projected for the near future.

Returning to our discussion of figure 1, it should be noted that while alternative schemes are possible, an important feature is observed to be characteristic of all. The various steps proposed in each of the alternative diagrams fall into two general categories: a series of preliminary processes leading to the production of non-specific precursors, and a second stage during which final structure and specificity is conferred upon the antigen.

From this point of view the present work assumes broader interest, since it suggests that the synthesis of proteins, whose specificity we have been detecting in our immunological tests, occurs in two gene-controlled stages. It

mechanisms in connection with the present case, but it is interesting to note that such two-step mechanisms for protein synthesis have been proposed on entirely different grounds by a number of other workers. Thus, SPIEGELMAN (1948), on the basis of his studies of adaptive enzymes, proposes a pool of protein precursors, whose elaboration comprises the first stage of protein synthesis, and the conversion of these into active enzyme through the action of plasmagenes. While we believe that our case exhibits only nuclear control, the similarity of the two proposals is apparent. Along different lines, HAUROWITZ (1950) suggests that the first stage of protein synthesis consists of the construction of the polypeptide chain on a two-dimensional template, and that during the second stage the chain is folded on a three-dimensional template in such a manner as to result in a surface configuration which is responsible for the specificity of the final protein molecule. Again, the details differ but the two-stage concept is present. Finally, NORTHROP (1948) proposes the production of proteinogens as a first step and their autocatalytic conversion into final protein under the influence of pre-existing protein as a second step. That such similar concepts have been developed from several different approaches is rather striking and certainly merits further attention. For this purpose we are currently examining the validity of the two-stage concept in connection with the genetic control of antigenic specificity in a second series of stocks of *Drosophila melanogaster*, and in connection with the genetic and environmental control of the synthesis and specificity of tyrosinase in strain 15300, *Neurospora crassa*.

SUMMARY

Immunogenetic studies of a series of *Drosophila melanogaster* stocks consisting of an isogenic type stock and co-isogenic ruby, vermilion and ruby-vermilion stocks have previously been reported. These studies demonstrated a specific antigenic fraction (Antigen 1) present in the Isogenic stock but not in the mutant stocks, indicating that this antigenic specificity is the product of interaction between the wild alleles at the ruby and vermilion loci. Two additional series of stocks have now been analyzed. Series 1 was derived by crossing the original ruby-vermilion to the original Isogenic and extracting new type, ruby, vermilion and ruby-vermilion stocks. It provides a test for the possibility that the antigenic differences between the original stocks were due to undetected genetic differences other than those at the ruby and vermilion loci. The antigenic pattern of Series 1 is identical with that of the original series, thus confirming the conclusion that Antigen 1 is the interaction product of the wild alleles of ruby and vermilion. Series 2 was derived by outcrossing each of the original stocks to an unrelated, non-isogenic wild stock. This series allows a test of the antigenic effects of mutation at the ruby and vermilion loci on a different genetic background. Antigen 1 is not found in any of the stocks of this series, indicating that in the original series one or more loci in addition to ruby and vermilion participate in the determinaton of its specificity. The relation of these results to the problem of the genetic control of protein synthesis and specificity is discussed.

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ADDENDUM

PARTIAL CHEMICAL ISOLATION AND CHARACTERIZATION OF ANTIGEN 1

PAUL FINKELSTEIN AND ALLEN S. FOX

Before the disappearance of Antigen 1 from the type 1 stock a chemical isolation was attempted. Since this work was terminated by the loss of Antigen 1, it seems important to record the progress made.

The material used in the isolation attempts consisted of homogenates prepared in a fashion identical with those used in the immunological work (Fox 1949b). Briefly, mass cultures of type 1 were grown on standard corn-meal agar medium unseeded with living yeast, the flies collected, starved, lyophilized without prior killing, and homogenized in an all-glass homogenizer with 0.85% NaCl buffered at pH 7.4 with 0.005 M phosphate (gram dry weight per 50 ml buffer). Prior to fractionation the homogenates were centrifuged, the clear, colored supernates extracted with cold ether by shaking gently in a separatory funnel and recentrifuged.

The ultraviolet and visible absorption spectrum of the final supernate was similar in all respects to that reported for wild type by previous investigators of Drosophila eye pigments. Most of the visible color was removable by dialysis against pH 7 buffer (0.02 M phosphate plus 0.14 M NaCl) but a faint yellow color was still observed in concentrated solutions. The absorption spectrum of the dialyzed extracts was that of a typical protein (peak at 280 mμ and minimum at 250 mμ), and the extracts gave the usual protein tests. In addition, however, there was a small peak at 410 mμ, probably accounting for the remaining visible color and suggesting the presence of a pigment conjugated to protein and previously unreported.

During all subsequent steps of concentration and fractionation the effect of each operation on Antigen 1 was measured by adjusting the protein concentration of the fraction being tested to that of the original material and testing its antigenic activity by means of precipitin tests with specific antisera to Antigen 1. Such tests of the dialyzed material indicated that Antigen 1 was not dialyzable. Furthermore, dialysis significantly increased the antigenic activity per unit solution. This may indicate the removal of an inhibitory low molecular weight material.

Electrophoresis of such dialyzed extracts in pH 7 buffer indicated the presence of four or five components. At this pH all of these exhibited a negative charge. The fastest moving component was colored (i.e., contained the pigment mentioned above), but antigenic tests following separation indicated it to be devoid of Antigen 1.

Attempts to concentrate the proteins of the dialyzed extracts in the presence of salts resulted in antigenic inactivation. Tests indicated that NaCl concentrations of 0.5 M and above destroyed antigenic activity. Concentration was satisfactorily accomplished by pervaporation at 5°C after a preliminary dialysis against distilled water.

In a salt-free solution of extract containing Antigen 1 about 70% of the protein was insoluble. This precipitate could be redissolved in the pH 7 buffer, but antigenic test indicated that the supernate of such precipitation was enriched in Antigen 1. Strikingly, similar treatment of the mutant stocks of series 1 yielded no such precipitate.

Alcohol fractionation at −5°C in pH 7 buffer allowed a concentration of Antigen 1 as follows: The extract was brought to 28.5% alcohol (volume/volume) and the precipitate was removed. This precipitate exhibited little activity. Then the alcohol concentration was raised to 44.4% and the resulting precipitate again removed. This precipitate proved to be greatly enriched in Antigen 1 and probably contained most of the antigen present in the original extract since the precipitate formed when the alcohol concentration was further raised to 50% exhibited very little activity. Thus, while Antigen 1 could not be completely characterized it was undoubtedly protein, some of the properties of which are indicated by these results.

THE EFFECTS OF INVERSIONS ON CROSSING OVER IN DROSOPHILA ROBUSTA

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FACTORS which affect the amount of genetic recombination in a species are of great importance in determining its evolutionary pattern. Natural selection, operating on the genetic system, in many cases tends to strike a balance between too much recombination, which may break up adaptive complexes of genes, and too little, which leads to specialization and evolutionary rigidity. The process of crossing over, as one of the primary effectors of recombination, occupies a position of central importance primarily because the intensity of its effect may, in an evolutionary sense, be more easily modified genetically than that of segregation or syngamy.

Extensive information has accumulated which shows that the most significant effect of an inversion of a chromosome segment is to reduce or effectively prevent recombination between sections of chromosomes. In organisms where the conditions for the persistence of inversions in natural populations are fulfilled, the gene complexes so isolated from recombination may react as units to natural selection, or may be held in equilibrium by coadaptation, so that the species displays the phenomenon of balanced polymorphism.

In most discussions of the effects of inversions on the recombination system, emphasis has been placed on their effects in greatly reducing recombination within the limits of the inverted segment itself (e.g., STURTEVANT and BEADLE 1936). On the other hand, considerable information exists that inversion heterozygosity not only affects crossing over outside the limits of the inversion in the same chromosome, but also under some circumstances profoundly influences crossing over in the other chromosomes (SCHULTZ and REDFIELD, in MORGAN, BRIDGES and SCHULTZ 1930; SCHULTZ and REDFIELD 1951; STEINBERG 1936, 1937; STEINBERG and FRASER 1944; KOMAI and TAKAKU 1940, 1942). These facts have important implications for any thorough understanding of the dynamics of inversions in natural populations.

The present study represents an attempt to discover the major outlines of the effects of inversions on crossing over in *Drosophila robusta* STURTEVANT. This species has been the object of considerable study of natural populations (CARSON and STALKER 1947; STALKER and CARSON 1947; LEVITAN 1951). These populations show a large amount of natural chromosomal polymorphism due to the presence of inverted sections. The inversions have become established in nature under the influence of natural selection. It is, therefore, of considerable interest to compare their effects on crossing over with those in other species, and to reach a better understanding, even if only in a general way, of the recombination pattern imposed upon the species by their presence.

MATERIALS AND METHODS

Drosophila robusta has a metaphase chromosome group which consists of three pairs of V-shaped chromosomes in addition to a pair of dots. These entities may be easily recognized in the salivary gland nuclei inasmuch as the two arms of the V-shaped chromosomes are commonly connected through the chromocenter, which is small and diffuse in this species. These chromosomes, together with the different gene arrangements used in this study are dia-

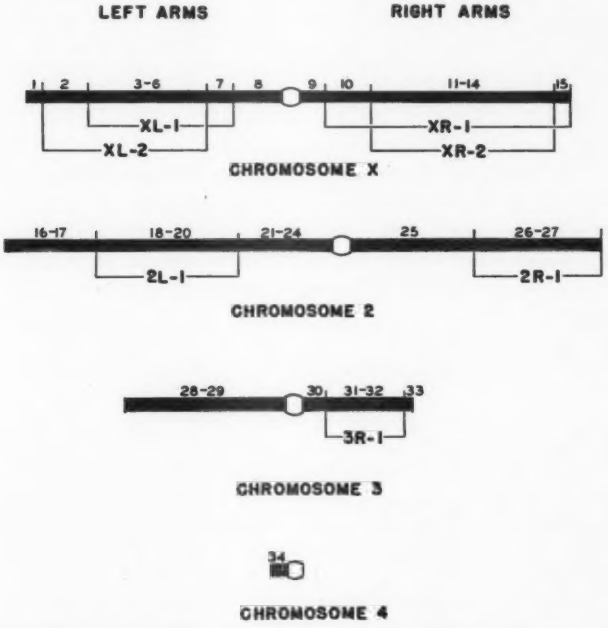

FIGURE 1.—Diagrams of the relative length of the euchromatic sections of the salivary gland chromosomes of *D. robusta*. The numbers above the dark lines, which represent the standard arrangement of the euchromatin, refer to the map regions (see CARSON and STALKER 1947). The inverted sections studied in this paper are bracketed; the light areas represent the heterochromatic bases of the chromosome arms and the centromeres.

grammed in figure 1. More detailed descriptions of the gene arrangements of this species, including the less common ones not used in this study, may be found in CARSON and STALKER (1947). The arbitrary "standard" gene sequences of *D. robusta* are designated XL, XR, 2L, 2R, 3L and 3R and it will be noted in figure 1 that there are alternative arrangements, due to inverted segments, in all chromosome arms except the left arm of chromosome 3. The small 4th chromosome has not been considered in this study.

In order to define the position and extent of the inversions used, camera

lucida tracings of a large number of heterozygotes were made from salivary gland chromosome smears; chromosomes were selected which showed the minimum amount of distortion. The relative lengths of each section of the chromosome were then measured with a map-measure and the average length of each section calculated in terms of its percent of the total chromosome length. The proportionate lengths of each chromosome arm of the set have been calculated relative to the length of the arms of the second chromosome. This was done by making tracings of XL, for example, along with tracings of 2L, 2R or both from the same nucleus. The resulting proportions are carefully reproduced in figure 1, and give a reasonably accurate picture of the positions of the various inversions and the relative sizes of the euchromatic sections of the chromosomes as observed in the salivary gland cells.

The following shorthand method of writing the structural karyotype of an individual female of *D. robusta* has been adopted for use in this paper. The conditions in each chromosome are written as three sets of symbols, from left to right, following the order X, 2 and 3. The capital letter " S " is used to refer to the standard arrangement in each case and numerals are used to refer to the various alternative gene orders in that particular arm. Thus, the formula for an individual female homozygous for all standard arrangements would be written:

$$\frac{\begin{matrix}X\\SS\end{matrix}}{SS} \qquad \frac{\begin{matrix}2\\SS\end{matrix}}{SS} \qquad \frac{\begin{matrix}3R\\S\end{matrix}}{S}$$

In the symbols for chromosomes X and 2, the S's at the left in each case refer to the gene arrangements in the left arms, the centromeres are inferred as being in the center, so that the S's on the right refer to the conditions in the right arms. In chromosome 3, the left arm is omitted in the formula, inasmuch as in none of the structural karyotypes used in this study was it involved in an inversion. Following this scheme, any combination may be simply symbolized and visualized. For example, an individual heterozygous for the four inversions XL/XL-1, XR/XR, 2L/2L-1, 2R/2R-1, 3R/3R-1 may be written as follows:

$$\frac{\begin{matrix}X\\SS\end{matrix}}{1S} \qquad \frac{\begin{matrix}2\\S1\end{matrix}}{1S} \qquad \frac{\begin{matrix}3\\S\end{matrix}}{1}$$

This formula gives at a glance the further information that in this case the arrangements in the second chromosome bear a " repulsion " relationship to one another rather than " coupling," which would be written:

$$\frac{\begin{matrix}2\\SS\end{matrix}}{11}$$

Most of the strains of *D. robusta* used in this study were derived from single wild-caught females. The gene arrangements present for each arm of each

chromosome were in most cases determined by making acetocarmine smears of the salivary glands of offspring of the original wild female. In a number of cases, it was necessary to select for certain arrangements in a stock so that a desired structural karyotype could be obtained. In this selective procedure, twelve or fifteen virgin females were taken at random from a stock and mated individually to single males from the same stock. Salivary gland chromosome smears of the offspring of each of these pair matings were then examined and the pairs transmitting the desired combinations were selected and new stocks established from these. Several repetitions of this process were sometimes necessary to obtain the desired combinations.

By making appropriate matings between individuals from such stocks, F_1 females of a large variety of inversion karyotypes could be obtained. Crossing over in this study was for the most part detected cytologically in salivary gland chromosome smears of the offspring of such selected females, which were mated to males homozygous for gene arrangement. For example, from a cross such as the following:

$$\begin{array}{c} \text{female} \\ \dfrac{\begin{matrix}2\\S1\end{matrix}}{1S} \end{array} \times \begin{array}{c} \text{male} \\ \dfrac{\begin{matrix}2\\SS\end{matrix}}{SS} \end{array}$$

the non-crossovers from chromosome 2 would be:

$$\frac{S1}{SS} \quad \text{and} \quad \frac{1S}{SS}$$

Individuals which had received a gamete from the female parent in which crossing over had occurred in the central region of the chromosome between the two inversions would be:

$$\frac{SS}{SS} \quad \text{and} \quad \frac{11}{SS}$$

Because of the favorableness of the chromosomes of this species both heterozygotes and homozygotes can be quickly and accurately determined. In practice, a single gland from each larva was used, and ten such glands smeared in two rows of five each under a single 22×40-mm coverslip. The rapidity of this method has made cytological detection of crossovers possible at a reasonably quantitative level.

In all of the tests here reported, data on the offspring of single females were recorded. The female was transferred to a new vial every two days and the larvae to be smeared were selected at random as they matured in the older vials. The data obtained on each day were kept separately. In general, smears were made from larvae which hatched from eggs oviposited over the first ten days of active egg-laying by young females. The above precautions were taken to minimize possible effects of the age of the female on crossing over. Under these conditions there was no pronounced or consistent age effect and the

temporal results have therefore been combined in the tables. All experiments were carried out at 25 ± 1°C.

OBSERVATIONS

Crossing over in the central region of chromosome 2 under various conditions of structural homo- and heterozygosity in chromosomes X and 3. In these experiments, the second chromosome configuration S 1/1 S was selected as a test object and crossing over in the section of the chromosome between the

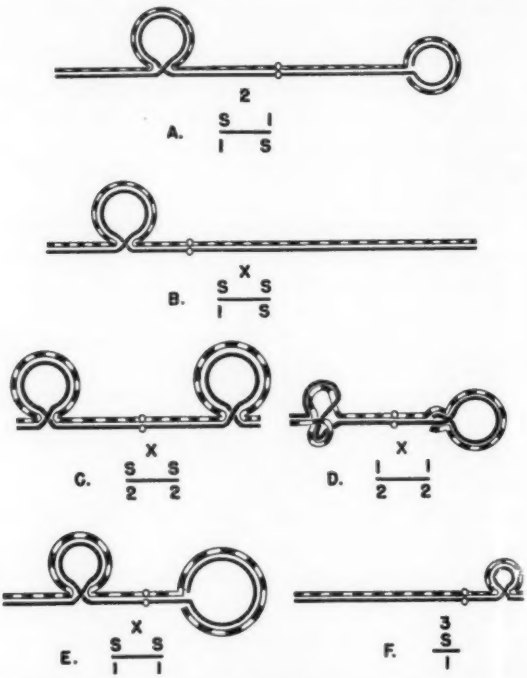

FIGURE 2.—Diagrams of the synaptic configurations, as observed in the salivary glands, formed by the various structural heterozygotes considered in tables 1, 2 and 3. The symbols below the figures are explained in the text.

inversions was studied under varying conditions of gene sequence in chromosomes X and 3. The cytological method for the detection of crossing over permits the observation of any crossovers which occur between the inversions in the second chromosome configuration S 1/1 S, that is, in regions 21–25 (see chromosome 2, figure 1). These regions together make up 37.6% of the total euchromatic length of the chromosome. This, of course, represents a minimum figure for the length of chromosome available for detected crossovers, inasmuch as it does not include the heterochromatin at the bases of the two arms. A dia-

gram of the double loop configuration formed by S 1/1 S, as seen in the salivary gland cell, is given in figure 2, A. The heterozygous configurations formed by the inversions in chromosomes X and 3, which have been used in various combinations with the above, are diagrammed in figure 2, B–F.

Table 1 shows the results obtained from a study of crossing over in the test configuration of chromosome 2 when there are (a) no inversion loops in X

TABLE 1

Crossing over in the central part (regions 21–25) of the doubly heterozygous chromosome 2, S1/1S, under varying conditions of homozygosity and single heterozygosity in chromosomes X and 3.

Expt. No.	Conditions in chromosome		No. of females	No. of F_1 larvae smeared	Percent of recombination
	X	3			
1	11 / 11	S / S	24	467	0.0
2	11	S / 1	3	304	0.0
3	22 / 22	1 / 1	1	100	0.0
4	22	S / 1	3	225	0.4
5A & B	SS / SS	S / S	(5A) 1	166	0.6
			(5B*) 4	589	4.2
6	S1 / S1	S / S	2	55	0.0
7	S1 / 11	S / S	3	250	0.0
8	11	S / 1	2	200	0.5

* Females in this experiment carried the inversions in the second chromosome in the coupling combination, SS/11.

and 3 (experiments 1, 3, 5 and 6) and (b) when X and 3 are singly heterozygous, either separately (experiments 2, 4 and 7) or coincidentally (experiment 8). Under these conditions, it is clear that crossing over in the test region of chromosome 2 is a very rare event. Except for the results in experiment 5B, which will be interpreted later, there are only 3 recombinations, or 0.2%, in 1767 F_1 larvae smeared.

When the X chromosome is structurally heterozygous in both arms, crossing

over in the test configuration is strikingly increased (table 2). Within each of the experiments reported in this table, the data are homogeneous (i.e., the p values are all above .05) except for experiment 13. In the latter case, χ^2 for the group of 12 females is 20.17, which, with 11 degrees of freedom, gives a p value of between .05 and .04. In view of the number of such tests made, no particular significance has been attached to this possible heterogeneity.

TABLE 2

Crossing over in the central part (regions 21–25) of the doubly heterozygous chromosome 2, S1/1S, when chromosome X is also doubly heterozygous.

Expt. No.	Conditions in chromosome		No. of females	No. of F_1 larvae smeared	Percent of recombination
	X	3			
9A & B	$\frac{SS}{11}$	$\frac{S}{S}$	(9A) 3	300	3.3
			(9B*) 5	400	5.3
			total 8	700	4.4
10		$\frac{S}{1}$	3	300	9.0
11		$\frac{1}{1}$	2	200	6.5
12	$\frac{11}{22}$	$\frac{S}{S}$	4	332	19.3
13		$\frac{S}{1}$	12	1150	34.3
14		$\frac{1}{1}$	4	400	7.5
15*	$\frac{SS}{22}$	$\frac{S}{S}$	3	300	12.0
16		$\frac{S}{1}$	6	473	33.8

* Females in this experiment carried the inversions in the second chromosome in the coupling combination, SS/11.

Inspection of the data reveals that the boosting effect of certain X chromosome double heterozygotes is stronger than others. Thus in experiments 9 and 10 (table 2), we observe that the X configuration SS/11 exerts a boosting effect on crossing over in chromosome 2 (compare with experiments 7 and 8, table 1). This effect, however, is considerably less than that of the X chromosome configurations which are heterozygous for XL–2 : XR–2 (experiments 12–13; 15–16).

Conditions in the third chromosome also exert an important effect. In the first place, females which are heterozygous S/1 in the third chromosome (experiments 10, 13 and 16) show more crossing over than when chromosome 3 is homozygous S/S. These differences are significant: for experiment 9 vs. experiment 10, χ^2 is 8.03, p less than .01; for experiment 15 vs. experiment 16, χ^2 is 46.04, p negligible. Despite the suspicion of heterogeneity within experiment 13, there is no question that the results differ widely from those obtained in experiment 12.

It is thus clear that double structural heterozygosity in the X chromosome greatly increases crossing over in chromosome 2 and that this effect is intensified by heterozygosity in the right arm of chromosome 3.

A second and very important fact is revealed by a comparison of experiment 11 with experiment 12 and 14 with 15 (table 2). In each of these pairs of experiments, the inversion karyotypes are structurally the same; they differ only in the nature of the 3rd chromosome homozygote (either 1/1 or S/S). Nevertheless, in both cases the amount of crossing over is significantly less in homozygous 1/1 individuals. For experiments 11 and 12, χ^2 is 16.49, which, with one degree of freedom gives a negligible p value; for experiments 14 and 15, χ^2 is 4.05, giving a p value of .05–.02. These, then, represent instances where females with karyotypes which are the same as far as the presence of inversion loops goes, show significant differences in crossing over. Such differences must be due to genetic differences apart from the presence or absence of inversion loops.

There is further evidence which supports the above suggestion of a genic effect. It will be noted (table 1) that experiment 5A differs from 5B, although the karyotypes are the same. This case is not entirely satisfactory inasmuch as the females in experiment 5B carried the test inversions in coupling, rather than in the usual repulsion. *A priori*, one would not expect any differential effect on crossing over between coupling and repulsion inversion figures, and the similarity of the results in experiments 9A and 9B (table 2) supports this conclusion. It is therefore suggestive that the differences between 5A and 5B are due to genic differences. The females used in experiments 5 and 6, unlike the rest, were wild females collected in nature and thus represent a rather more heterogeneous sample than the inbred laboratory strains used in the other experiments.

In experiments of this kind, where selection and backcrossing must be used in order to obtain the desired inversion karyotypes, it has not been possible to control in a systematic way the genetic background of the females tested. Nonetheless, in a number of cases the females for the experiments were selected from among a segregating group of sibs. Thus, for example, females in the following experiments were sibs: 14 and 16; 9B and 15, 9A, 10, 12 and some of the females in 1, 3, 4 and 11. In each of these experiments females with identical structural karyotypes showed no significant differences, while the interkaryotype differences were large. This suggests a prominent effect of the structural karyotype.

The above results may be summed up as follows. When chromosome 2 has

an inversion in each arm, and crossing over in the central section is observed, it is found that recombination is very low unless there is a considerable degree of structural heterozygosity in the other chromosomes. Double heterozygosity in chromosome X raises crossing over in chromosome 2 to the point where an affect of heterozygosity in the right arm of chromosome 3 also can be observed. In addition to these apparently direct interchromosomal effects of structural heterozygosity, clear differences in crossing over between individuals of similar inversion karyotypes indicate that there is also a genic effect.

Crossing over in the X chromosome. Crossing over in the X chromosome under varying conditions of structural hetero- and homozygosity has been studied with the use of two sex-linked recessive genes, echinus eye (*e*) and scarlet eye (*st*). This stock was kindly supplied by DR. A. H. STURTEVANT. Salivary gland chromosome smears showed the X chromosome of this strain to be homozygous XL–1 : XR–1.

The location of echinus and scarlet. A number of experiments were done in an attempt to determine the positions of *e* and *st* in the X chromosome with respect to the positions of the relatively inverted segments. From these experiments, the best estimate of the positions of these genes which can be given is that echinus is located in the left arm distal to region 8 and that scarlet is located in the right arm distal to region 9 (see figure 1).

The above conclusion was reached as follows. In females homozygous XL–1 : XR–1 and simultaneously *e st*/++ (experiment 17, table 3), the two genes segregate at random. This indicates that they are either in separate arms or are at least 50 units apart in the same arm. The females in this experiment, and also in experiment 18, were unavoidably heterozygous for gene arrangement 2L–3 (see CARSON and STALKER 1947); in view of experiments to be reported later, however, this probably has no appreciable effect on crossing over in an X chromosome homozygous for gene arrangement.

Echinus and scarlet also segregate at random when the X chromosome is *e st*/++ and simultaneously XR/XR–1 (experiment 18, table 3). It will be noted that this inversion covers a large percentage of the euchromatin in the right arm and is terminal (figure 1 and figure 2, E, part to the right of the centromere). Six echinus and six scarlet crossover males were tested for gene arrangement by crossing them individually to females homozygous for gene arrangement. Each of the scarlet individuals was XL–1 : XR–1 and each of the echinus individuals was XL–1 : XR, that is, all were crossovers between *e* and the XR–1 inversion. These individuals are considered to arise as simple single crossovers. From these results, it will be seen that *e* cannot be located within the XR–1 inversion and, further, must be located to the left of *st*.

Similar results are obtained when the X chromosome is *e st*/++ and simultaneously XL/XL–1 (experiments 7 and 19, table 3); under these conditions *e* and *st* again segregate at random. Again, six crossover males of each type from experiment 19 were tested and as all six scarlet individuals were also crossovers between XL–1 and *st*, the latter gene cannot be located within XL–1. Because echinus must be to the left of scarlet, the latter cannot be

TABLE 3

Crossing over between e (echinus) and st (scarlet) in the X chromosome under varying conditions of structural heterozygosity.

Expt. No.	Structural karyotype of tested female			Total counted	Percent crossovers between *e* and *st*	Karyotypes of recombined X chromosomes	Percent recombination in central region of chromosome 2 (from tables 1 & 2)
	X	2	3R				
17	1++1 / 1 *e st* 1	1S / 3S	S / S	5638	49.5	*e*: 11 *st*: 11	....
1	1++1 / 1++1	S1 / 1S	S / S	....	....	....	0.0
18	1++S / 1 *e st* 1	1S / 3S	S / S	2111	48.8	*e*: 1S *st*: 11	....
7	S++1 / 1 *e st* 1	S1 / 1S	S / S	1155	48.1	(Not tested)	0.0
19	S++1 / 1 *e st* 1	1S / 1S	S / S	319	48.6	*e*: 11 *st*: S1	....
20	S++1 / 1 *e st* 1	Variable*	S / S	4166	47.2	(Not tested)	....
12	1 *e st* 1 / 2++2	S1 / 1S	S / S	1458	0.00	(None obtained)	19.3
13	1 *e st* 1 / 2++2	S1 / 1S	S / 1	1010	0.29	*st*: 3 individuals; abnormal; sterile	34.3
21	1 *e st* 1 / 2++2	1S / 1S	S / 1	2272	0.00	(None obtained)	....
9A	S++S / 1 *e st* 1	S1 / 1S	S / S	3512	0.03	*st*: 11	3.3
22	S++S / 1 *e st* 1	Variable†	S / S	13260	0.02	*e*: SS *e*: 11	....
10	S++S / 1 *e st* 1	S1 / 1S	S / 1	1301	0.77	*e*: 11 *st*: SS	9.0

* 1S/1S, S1/1S or SS/1S.

† SS/1S or SS/S1.

located to the left of the distal break of XL–1 (regions 1 and 2), because a crossover between *e* and *st* in this position could not also be a crossover between *st* and XL–1.

In the experiments with the doubly heterozygous X chromosome configuration S (++) S/1 (*e st*) 1 (experiments 9A, 22 and 10, table 3) there are very few crossovers between *e* and *st* (14 out of 18,073, or 0.08%). Nine of these individuals were tested for gene arrangement in the X; four were *e*: 1 1, three were *st*: S S; one was *e*: S S; one was *st*: 1 1. This result is crucial inasmuch as none of these chromosomes is also a recombination between the inversions. This means that, whether they were formed by single or by double crossing over, in no case did a single crossover occur in the central part of the chromosome (regions 8–9) between the inversions. If this event had occurred, the sequences S 1 or 1 S would have been recovered. Thus, *st* cannot be located in the center section of the chromosome, because on this assumption, the only way that the three *st*: S S and the four *e*: 1 1 chromatids could form would be by double crossing over in this center section. This would require the occurrence of double crossing over seven times in a section where no singles have been detected. By elimination, therefore, *st* must be located in the right arm, within the XR–1 inversion (regions 10–15). Likewise, if *e* were in the center section, the recovery of the *e*: S S and *st*: 1 1 chromosomes could only be explained by double crossing over in the center section, effectively removing *e* to S S. It is therefore concluded that *e* is located in the left arm of the X, to the left of region 8.

On the above basis, the crossover individuals tested from experiments 9A, 22 and 10 (table 3) would be explained as follows: The *e*: 1 1 and *st*: S S individuals arose through double crossing over within the large inversion loop in the right arm. The *e*: S S and *st*: 1 1 individuals could have arisen either through a single crossover between *e* and XL–1, if *e* is distal to the latter, or through double crossing over within inversion XL–1, if *e* is located there. The latter hypothesis is considered the more likely, due to the very great rarity of crossovers between *e* and XL–1.

Concurrent crossing over in chromosomes X and 2. As has been shown above, crossing over in the central region of the doubly heterozygous chromosome 2 is very low when the X chromosome is structurally homozygous or singly heterozygous. The information given by experiment 7 (table 3) shows that under these conditions crossovers are abundant in the X chromosome, and no difference is detectable when chromosome 2 is homozygous (experiment 19) or singly heterozygous (experiments 17 and 18). When, however, chromosome X is doubly heterozygous, either 1 1/2 2 or S S/1 1, we have seen previously that crossing over in the central region of chromosome 2 is greatly increased. It may now be seen that this increase is correlated with a very pronounced blocking of crossing over in chromosome X by the presence of an inversion in each arm. This effect is strikingly emphasized by the fact that from the doubly heterozygous X chromosomes studied, not a single case of crossing over in the central region of the chromosome has been observed

(experiments 12, 21, 9A, 22 and 10, table 3). The three *st* crossovers in experiment 13, table 3 represent possible exceptions; as they were sterile, they could not be tested for gene arrangement.

The rare crossovers in the X chromosome appear primarily in experiments where the second chromosome was doubly heterozygous and the right arm of the third chromosome was likewise heterozygous. This is indicated by a comparison of the results in experiment 13 with those in experiments 12 and 21, table 3 and the comparison of experiment 10 (10 double crossovers in 1301) with experiments 9A and 22 (4 crossovers, at least one of which was a double, in 16,772). This suggests that such crossovers are "forced out" by the residual structural heterozygosity. The complex configuration in the X formed by the double heterozygote $\underline{1\,1}/\underline{2\,2}$ (see figure 2, D: overlapping inversions in the left arm and included inversions in the right) appears to be more resistant to such forcing. The only crossovers which were obtained from it were weak, abnormal, sterile females. This suggests, as does the fact that they were scarlet, that they arose by a crossover within the included portion of the figure in the right arm of the X, an event which would result in duplication-deficiency. This is further supported by the fact that among 51 F_1 larvae smeared from a wild female of the constitution:

X	2	3
S 1	S S	S
1 2	1 S	S

there were no crossovers between the inversions in the X, but one larva carried a chromosome which was duplication region 10 and deficiency region 15 in the right arm. This is the expected result following an exchange within the included portion of this right arm figure.

The facts presented above suggest that reductions or suppressions of crossing over in the X chromosome may bear some relationship to the degree of complexity of the configuration involved. Thus, the X configuration $\underline{1\,1}/\underline{2\,2}$ has a stronger boosting effect on crossing over in chromosome 2 than does the simpler configuration $\underline{S\,S}/\underline{1\,1}$. Correlatively, the former appears to permit fewer crossovers in the X itself.

Despite the above considerations, however, several facts suggest that these interrelationships are again not wholly structural. In the first place, the structural heterozygote $\underline{S\,S}/\underline{2\,2}$, which is simpler than the others studied (see figure 2, C) and leaves a long central area, amounting to about 29% of the euchromatin, available for crossing over, has the same effect on the doubly heterozygous chromosome 2 as does the complex figure $\underline{1\,1}/\underline{2\,2}$. As these differ strikingly in this respect from the heterozygote $\underline{S\,S}/\underline{1\,1}$, one is led to consider that some peculiar property of the combination XL–2: XR–2 is involved rather than the purely structural inversion figures formed. XL–2 and XR–2 heretofore have been suspected of showing unusual behavior. A highly significant association exists between these two arrangements in natural populations (CARSON and STALKER 1949) and it has been suggested that XR–2

acts as a " protector " of XL–2, inasmuch as the latter has only in several rare instances been observed to occur in natural populations apart from XR–2 in the same chromosome.

In the laboratory, no certain instance of the separation of the XL–2 : XR–2 complex by crossing over has been observed. This has been studied genetically, in heterozygotes with 1 (*e st*) 1 and cytologically in heterozygotes with S S. The latter was done by NELSON (1951) who examined 414 F_1 larvae of females which were heterozygous S S/2 2 without finding a single recombination. Unfortunately, the difficulty of extracting *e* and *st* from their association with XL–1 : XR–1 has hampered the usefulness of these genes in the study of crossing over in various X chromosome heterozygotes.

The analysis of the interchromosomal effects of inversions is probably affected in some measure by the following considerations. When a V-shaped chromosome is heterozygous for an inversion in each arm, there is likely to be a considerable number of double crossovers which are made up of singles within each inversion loop. Half of these will lead to the death of the zygote (or to X0 males, in the case of the X chromosome) rather than selective elimination of the crossover strands. If these events were to occur in the X chromosome, they would be expected to result in an increase of recovered crossovers in chromosome 2, not because more crossovers occurred but because more non-crossovers died.

From the data presented in this paper, it is not possible to estimate the magnitude of this effect. Certain of the results given in table 2, however, have some bearing on this question and make it seem unlikely that this effect is a prominent one. It will be noted in figures 1 and 2 that the X chromosome double heterozygotes S S/1 1 and S S/2 2 form loops which are rather similar in size. XR–1 is the largest, covering 46.3% of the euchromatin; the figures for the others are: XL–1, 27.1%; XL–2, 30.8%; XR–2, 34.6%. When the results of experiments 10 and 16 (table 2) are compared, it appears to be highly unlikely that the very large difference in recombination frequency observed in the second chromosome could be due to the more frequent occurrence of the required types of double crossovers in the X configuration S S/2 2 rather than in S S/1 1. The results in experiments 9 and 15 (table 2) may be similarly compared. Under any circumstances, moreover, the *effective* frequency of recombination in chromosome 2, in a population sense, would be increased.

The major effects of the inversions of *D. robusta* on crossing over, as revealed in the above experiments, may be outlined as follows.

1. There is an apparent intrachromosomal suppression of crossing over in the central section of a chromosome which has an inversion in each arm. This suppression is absolute in the X chromosome cases studied but is less pronounced in the case of the second chromosome.

2. There is interchromosomal intensification of crossing over in certain chromosome sections when a certain level of structural heterozygosity is reached. The double heterozygotes in chromosome 2 and X interact on each

other. Central intrachromosomal suppression in chromosome 2 is weakened by the interchromosomal boosting effects of double heterozygotes in the X. Double heterozygotes in chromosome 2, however, appear to be less effective in intensifying crossing over in chromosome X.

3. Structural heterozygosity in chromosome 3 intensifies the above interchromosomal effects.

4. Certain of the X chromosome double heterozygotes, especially those involving XL–2 : XR–2, have a stronger interchromosomal effect than others.

5. The amount of crossing over in a given section is not wholly dependent on structural conditions in either the same chromosome or in other chromosomes. Thus a genic influence may affect in a minor way the amount of crossing over as conditioned by the structural heterozygosity.

As a means of visualizing the results, the following crude scheme is suggested; it is used for exposition only, and is not to be taken as having an explanatory value. Assume that a nucleus about to undergo meiosis has a certain total quota of crossovers available to it. The size of this quota may or may not be fixed; it may be affected by structural conditions or genic factors or both, and it may differ from individual to individual. These crossovers may be visualized as forming in those sections of the chromosomes where the least resistance to crossover formation is met. Thus in an individual of the formula:

X	2	3
1 1	S 1	S
1 1	1 S	S

we know that crossing over is largely suppressed between the inversions in chromosome 2; we would then visualize increased crossing over in less resistant sections, possibly in those chromosome arms homozygous for gene arrangement. In a highly heterozygous individual, such as:

X	2	3
1 1	S 1	S
2 2	1 S	1

the crossovers meet strong interference due to inter-inversion suppressions and possibly intra- and para-inversion suppression. We may visualize crossing over as occurring, maximally, perhaps, in the one free homozygous arm (3L); the remainder of the quota, however, must be distributed in sections where crossing over meets with more resistance. It is suggested that these sections differ in their degree of resistance so that, for instance, the central section of the second chromosome, a region of relatively low resistance, now acquires more crossovers than it would when the inversions are absent in the other chromosomes.

DISCUSSION

The essential effects of heterozygous inversions on the frequency and pattern of crossing over fall into three interrelated categories and may be set forth as follows:

1. *Suppression of crossovers within an inverted section.* The definitive work of STURTEVANT and BEADLE (1936) on *D. melanogaster* shows that effective genetic recombination between relatively inverted chromosome segments is negligible, with the possible exception of very long inversions, in which a significant number of 2-strand double exchanges could occur. This blocking of recombination, however, is not due primarily to the suppression of exchanges initially, but rather to the selective elimination of most types of crossover strands from the definitive egg nucleus (see also CARSON 1946). Quite apart from this mechanical elimination of the products of crossing over, however, it is clear that inversions, especially short ones, do interfere to some extent with the actual formation of crossovers within the inverted segment. Thus from individuals heterozygous for the relatively short X-chromosome inversions delta-49 and scute-7, STURTEVANT and BEADLE recovered fewer 2-strand double exchanges than expected. This was not true for longer inversions such as scute-8. The critical length for such effects has not been determined and, indeed, other unspecified effects on crossing over may exist as properties of individual inversions. There is thus no reason to believe that the suppressive effect of an inversion on crossovers within it must necessarily be directly proportional to the length of that inversion.

2. *Suppression of crossing over distal or proximal to the inversion in the same chromosome.* STURTEVANT and BEADLE (1936) found that certain X chromosome inversions in *D. melanogaster* suppressed crossovers adjacent to them; these effects were generally greater distally (between the inversion and the free end of the chromosome) than proximally. Similar effects were found by DOBZHANSKY and EPLING (1948) for certain 3rd chromosome inversions in *D. pseudoobscura,* although in some cases proximal suppression was also strong. In the above cases, the inversions studied have one break point close to the distal end. That these cases of strong distal suppression may be mediated, at least in part, by this fact is indicated by the findings of KOMAI and TAKAKU (1940, 1942) in *D. virilis.* Although there is strong suppression proximally when two independent inversions are present in the rod-shaped X chromosome of this species, the distal end was affected only at a point close to the inversion, and crossing over was actually increased in the long free distal end. When an inversion is present in each arm of the V-shaped chromosomes X or 2 of *D. robusta,* crossovers appear to be strongly suppressed in the region between the inversions. Such suppression appears to be closely comparable to that found in the V-shaped autosomes of *D. melanogaster* when an inversion is present in each arm (e.g., Curly inversions in chromosome 2; Payne inversions in chromosome 3). A similar case has been described in the V-shaped X chromosome of species hybrids between *D. pseudoobscura* and *D. persimilis* (LANCEFIELD 1929; MACKNIGHT 1937).

3. *Intensification of crossing over in heterologous chromosomes.* Inversion heterozygosity in one chromosome, under many if not all conditions, increases crossing over in the other chromosomes. The effects are not confined to regions which are homozygous for gene arrangement; thus if other heterozygous inversions are present, the frequency of recoverable double crossovers

is increased under these conditions. These phenomena have been subjected to intensive study in *D. melanogaster* (SCHULTZ and REDFIELD in MORGAN, BRIDGES and SCHULTZ 1930; STEINBERG 1936, 1937; STEINBERG and FRASER 1944; SCHULTZ and REDFIELD 1951) and have been studied in *D. virilis* (KOMAI and TAKAKU 1940, 1942), *D. pseudoobscura* (LEVINE and DICKINSON 1952) and in hybrids between *D. pseudoobscura* and *D. persimilis* (MACKNIGHT 1937).

The results obtained with *D. robusta* are in close general agreement with previous findings. Because of the fact, however, that the test regions, such as the central portion of chromosome 2, are under the influence of suppressions by inversions in the same chromosome, a considerable degree of inversion heterozygosity is necessary for the observation of a strong interchromosomal effect. The results are nonetheless striking, and the test region showed an approximately 30-fold increase under some conditions. Insufficient genetic markers are available in *D. robusta* for the testing of chromosome regions in the way that is possible in *D. melanogaster,* and control crossover frequencies and patterns in structural homozygotes are not available. Such control frequencies, however, would have little meaning for most natural populations of this species, in which a structural homozygote is a rarity (CARSON and STALKER 1947, 1949). Conventional chromosome maps for species which show extensive inversion heterozygosity in natural populations are useful primarily as an artificial frame of reference and do not reflect the dynamics of recombination as it occurs under natural conditions.

The fact that certain X chromosome heterozygotes in *D. robusta* have a stronger boosting effect than others correlates well with the findings of STEINBERG and FRASER (1944) who found similar differences among a number of X chromosome inversions in *D. melanogaster*. SCHULTZ and REDFIELD (1951) have confirmed and extended these observations. The latter authors, furthermore, found differential effects of the X chromosome homozygotes scute-8/scute-8 and +/+ on crossing over in the 3rd chromosome; this recalls the observation that 3R/3R and 3R–1/3R–1 have similar differential effects in *D. robusta*. As STEINBERG and FRASER have pointed out, factors other than the length of the inversion, its position relative to the chromosome ends and probably also the number of crossovers permitted within and adjacent to it, are involved in the interchromosomal effects.

Not only do individual inversion heterozygotes and their homozygotes thus vary in their intensification effects elsewhere in the chromosome set, but also different regions of the affected chromosomes react differentially to the intensification stimulus. This has been repeatedly demonstrated for *D. melanogaster* and has been confirmed in *D. virilis*. In these cases the chromosome regions most sensitive to the boosting effects lie near the centromere and distally in the chromosome arm. These are regions where, in the absence of structural heterozygosity, crossing over appears to be relatively low.

The data reviewed above indicate that crossovers tend to be suppressed within and adjacent to heterozygous inversions and that this is directly correlated with an increase of crossing over elsewhere. This interplay of intra-

chromosomal suppression and interchromosomal intensification of crossing over has suggested to a number of authors (e.g., MATHER 1936) that, by and large, inversions do not drastically affect the total frequency of exchanges in a nucleus; suppression in one region is accompanied by an increase in another. One may recall the remark of MULLER, who likened this phenomenon to the reaction of a balloon; if it is pressed in at one point, it bulges out at another. In most cases, this appears to be at least approximately true.

These considerations have important implications for natural populations. Natural selection for co-adapted inversion heterozygotes in a species may operate with chromosome sections greater than that involved in an inversion if recombination outside its limits, or between adjacent inversions, is strongly suppressed. The case of the XL–2: XR–2 chromosome described in this paper is an extreme example of this; no certain recombinants between the left and right arm have been found in natural populations or in laboratory experiments, even where the situation has been forced by strong inversion heterozygosity in other chromosomes. The whole chromosome thus appears to function as a non-recombining unit.

Co-adaptation of inversion heterozygotes is a flexible system which permits the species to maintain adaptations to a number of different ecological niches (DOBZHANSKY 1950). This is accomplished, however, with the sacrifice of a certain amount of recombination. Although recombination through crossing over may occur freely between homologous chromosomes of like gene sequence, two alternative arrangements are effectively isolated from one another. As inversion heterozygotes accumulate in a species, its hereditary material becomes more and more a mosaic of such isolations. Heterozygous inversions, however, effectively shunt crossovers into other sections of the chromosome set, apparently primarily those which are not involved in inversions. Such a result would keep available for the species the possibility of evolutionary adjustment through a highly active recombination of genes in at least part of the chromosome set. The species may thus exploit the advantages of adaptive chromosomal polymorphism and at the same time retain the more conventional advantage of intense recombination. This powerful side effect of the inversion system would seem to be of special importance in the higher Diptera which, because of the elimination of crossing over in males, are highly dependent on crossing over in females for the release of concealed variability.

SUMMARY

1. The influence of naturally-occurring inversions on crossing over in *Drosophila robusta* has been investigated for a series of selected structural heterozygotes. Both cytological and genetic methods for the detection of crossing over have been employed.

2. When an inversion is present in each arm of a V-shaped chromosome, there is a strong intrachromosomal suppression of crossing over in the portion of the chromosome between the inversions. This suppression is absolute in the X chromosome cases studied but is less pronounced in the second chromosome.

3. Crossing over is intensified in certain chromosome sections when a relatively high degree of structural heterozygosity is reached. Thus double heterozygosity in chromosome X is correlated with a significant increase of crossing over in the section between the two second chromosome inversions. The doubly heterozygous condition in chromosome 2, however, appears to be much less effective in intensifying crossing over in the X chromosome.

4. Structural heterozygosity in the right arm of chromosome 3 intensifies the above interchromosomal effects.

5. Certain X chromosome double heterozygotes, especially those involving XL–2: XR–2, have a stronger boosting effect than others.

6. The amount of crossing over in a given section is not wholly dependent on structural conditions in either the same chromosome or in other chromosomes. A genic influence may thus affect in a minor way the pattern and intensity of crossing over as conditioned by the structural heterozygosity.

7. The interplay of intrachromosomal suppression and interchromosomal intensification of crossing over indicates that inversions do not greatly reduce the total frequency of exchanges. On the other hand, crossovers appear to be shunted principally into sections of the chromosome set which are not involved in inversions. The suggestion is made that in this way the species may exploit the advantages of adaptive chromosomal polymorphism and at the same time retain evolutionary plasticity through a high degree of recombination in structurally homozygous chromosome sections.

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THE DETECTION OF LINKAGE IN TETRAD ANALYSIS

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ESTABLISHING the existence of linkage between genes at two loci is a distinct problem from estimating map-distance once genes are known to be linked. This paper considers two questions that concern linkage detection in organisms with genetically analyzable tetrads. First, what criterion is best for establishing linkage when tetrad segregations are analyzed? And second, what are the advantages of tetrads compared to random single strands for indicating linkage?

CHOICE OF A VALID AND EFFICIENT CRITERION FOR LINKAGE DETECTION WITH TETRADS

In classical single-strand analysis, linkage between two genes is indicated when the ratio of recombination to non-recombination gametes is significantly less than equality. For organisms (chiefly lower plants) where all four products of a single meiosis can be recovered, similar criteria have been adopted for detecting linkage. Ratios of whole tetrads have rarely been used, but segregants collected as tetrads have instead been treated as populations of single strands (e.g., POMPER and BURKHOLDER 1949) or of half tetrads (e.g., WHITEHOUSE 1942) for purposes of determining if recombination : non-recombination numbers differ significantly from the 1 : 1 ratio expected with independent segregation. (By half tetrad is meant specifically a pair of complementary products, $AB + ab$ or $Ab + aB$, originating from the same tetrad.) When segregants that have been obtained as tetrads are treated in this way, two types of error may result. Linkage may either be indicated spuriously where none exists, or linkage may remain concealed that would become apparent if the data were treated more critically.

A dihybrid zygote (*AaBb*), from gametes *AB* and *ab*, can produce any of three possible tetrad types: parental ditype ($AB + AB + ab + ab$), non-parental ditype ($Ab + Ab + aB + aB$) and tetratype ($AB + Ab + aB + ab$). Observed numbers of these classes are the basic data of tetrad analysis. Parental ditype (PD) and non-parental ditype (NPD) tetrads are equally probable if genes are unlinked, and it will be shown that a significant departure from equality of these two classes is both a reliable and an efficient criterion for establishing linkage when tetrads are used. The advantages of ditype tetrad numbers will be discussed in connection with examples in table 1, where alternative methods are used to examine data from a variety of tetrad segregations.

Non-independence of component parts of tetrads in testing departures from random segregation. It is not correct to assume that the two half tetrads from a single meiotic segregation are independent with respect to their recombinant

TABLE 1

The significance of deviations from random segregation indicated by ratios of non-parental: parental ditype tetrads and of recombinant: parental half-tetrads.

Example	Observed tetrad numbers, PD:NPD:T	Half-tetrad numbers, R:P	Probability* that specified numerical ratio is a random deviation from 1:1			% recombination	Organism	Segregating characters	Reference
			Tetrad numbers, NPD:PD	Half-tetrad numbers, R:P	Half-tetrad numbers × 1/2, R:P				
1	2	3	4	5	6	7	8	9	10
1	40:30: 0	60:80	.14	.05	.14	43**	*Sphaerocarpus donnellii*	v, sex	Knapp 1936
2	51:45: 0	90:102	.30	.21	.30	47**	"	d, sex	" "
3	25:20: 0	40:50	.28	.17	.28	44**	*Neurospora tetrasperma*	d, mt	Dodge et al. 1945
4	11: 5: 1	11:23	.10	.03	.11	32**	Marchantia	fa, hy	Burgeff 1943
5	11: 3: 2	8:24	.03	<.01	.04	25	*Neurospora crassa*	35203, 35301	Houlahan et al. 1949
6	1: 3: 43	....	....	....	....	52	Sphaerocarpus	u, sex	Knapp 1936
7	50:59:413	....	....	....	....	51	"	c, sex	" "
8	44:28:119	175:207	.04	.06	.14	46	*Saccharomyces cerevisiae*	g, me	Lindegren 1949
9	14: 4: 43	51:71	.02	.04	.13	42	"	ma, pb	" "
10	17: 5: 45	55:79	<.01	.02	.09	41	"	pb, alpha	" "
11	34:17:110	144:178	.01	.03	.10	45	Glomerella	conidial A, B	Chilton et al. 1949
12	10: 2: 20	24:40	.02	.03	.11	38	*Dunaliella salina*	r, mt	Lerche 1937
13	9: 2: 15	19:33	.03	.04	.12	37	*Chlamydomonas Moewusii*	b, mt	Lewin 1950
14	8: 0: 11	11:27	.004	.007	.05	29	"	t, 1	" "
15	22: 6: 48	60:92	.002	.006	.04	39	*Neurospora crassa*	C94, C141	Haas et al. 1952
16	24: 7: 63	77:111	.002	.008	.05	41	"	C141, T1710	" "

* P-values (one-sided) were obtained using a table of normal deviates and applying Yates's correction for continuity (McMullen 1936), except for small values in examples 14–16, which are from Warwick's tables.

** Location in separate linkage groups is indicated by low T and high NPD frequencies.

or parental composition, either for linked or unlinked genes. To use actual numbers of half tetrads may therefore be to increase the population size arbitrarily above its proper value, with the result that excessive significance is attributed to deviations. This is a serious objection to using component tetrad parts for linkage detection.

The null hypothesis, independent segregation, predicts equal numbers of parental and non-parental ditype tetrads, but sets no limits to the proportion of tetratypes. Establishment of non-independence thus depends solely upon ditype tetrads, where the constitution of any portion defines the whole tetrad. For efficiency in detecting linkage, comparisons should be limited to ditype segregations. Sister halves from ditypes are identical, and may not be treated as statistically independent units. On the other hand, efficiency might be ignored, and the uninformative tetratypes included for analysis. Here also sister half tetrads may not be considered independent, because in crosses where ditype segregations are in excess over tetratypes a positive correlation exists between sister halves, and where tetratypes predominate, a negative correlation obtains.

The error from using actual numbers of half tetrads is most apparent when tetratype (T) segregations are infrequent or absent. In example 1, table 1, with 40 PD : 30 NPD : 0 T tetrads, the correct population size is 70, not 140, and in 70 segregations of independent genes, the probability of obtaining the observed ratio is clearly that of 40 : 30 occurring when 35 : 35 is expected, not 80 : 60 against an expected 70 : 70.

Observed numbers of parental and recombination single strands can validly be used for testing the null hypothesis of independent segregation where each strand originates from a separate meiosis, a situation approximated in classical methods with higher plants and animals. Half-tetrad numbers would be suitable units for comparison if each represented a separate segregation, as do the two strands recovered in attached-X Drosophila females, Habrobracon impaternate daughters or Bombyx mosaics from binucleate eggs. But where a sample of segregants consists of a series of whole tetrads, raw numbers of half (or quarter) tetrads are not valid for significance tests.

Half-tetrad ratios might perhaps be used if the numbers were first reduced so as not to attribute false significance to deviations. Such an adjustment was made by dividing actual half-tetrad numbers by two before computing the probabilities given in column 6 of table 1. Nothing is gained by computing adjusted half-tetrad ratios in this way, since they are less sensitive for detecting linkage, and more laborious to obtain, than ditype tetrad ratios.

Loss of sensitivity due to including tetratype segregations. A second major objection to using half- or quarter-tetrad numbers for linkage detection is that segregants from tetratype tetrads are then included, and these are irrelevant for establishing linkage. Ditype ratios are able to provide a more efficient and sensitive criterion of non-independence than half-tetrad or quarter-tetrad ratios because irrelevant segregations are excluded.

Unlinked genes may produce tetratypes in any proportion whatever, from zero to 100% of the total population (see WHITEHOUSE 1949, p. 231). The

frequency of tetratypes may be zero if two genes are at centromeres on different chromosomes (examples 1–3, table 1). At the other extreme, all segregations could result in tetratypes if one gene were at a centromere and another, unlinked, gene were separated from its centromere by an interval within which a single exchange always occurred, a condition approached in examples 6 and 7. No evidence for linkage is gained, therefore, by including data from tetratype segregations (as is done if half tetrads or single strands are used from the whole population of tetrads), and inclusion of numerous tetratypes may even conceal a significant departure from randomness that is apparent from the ratio of ditype tetrads (examples 8 ff.).

Failure of tetratype segregations to contribute evidence for linkage does not imply that they are unnecessary for other purposes. Tetratype frequencies are indeed essential for such operations as determining the intensity of linkage once its existence has been established, and it is important that all segregations, including tetratypes, be included when tetrad data are collected and published.

In particularly favorable cases tetratype frequencies may make it possible to establish from two-point segregations that genes are *not* linked, e. g., where the two ditype classes are numerous and equally frequent, but tetratypes are rare (examples 1–4, table 1; WHITEHOUSE 1949, p. 232). A very rigid criterion for non-linkage would be a significant deviation in excess of 1NPD:2T. This is the maximum ratio attainable for linked genes, barring chromatid interference, but would be realized only in the rare case where all exchanges occurred as doubles. A more practical test would be a significant deviation in excess of 1NPD:4T, which is the maximum ordinarily expected for genes distantly spaced in the same linkage group.

Application of ditype tetrad ratios to linkage detection. A variety of segregations have been gathered in table 1 and examined for evidence of linkage, using ditype tetrad ratios as a criterion. The results (column 4) stand in contrast to probabilities obtained when half-tetrad numbers are used (column 5). In examples 1–5 deviations of ditype tetrad numbers from 1:1 are less significant than those of half tetrads. The low probability values in column 5 are not applicable for linkage detection because sister half tetrads are not independent of one another, and use of these probabilities might lead one to accept linkage with false confidence. If the half-tetrad numbers are divided by two, so as to reduce the population from its inflated size, the new P-values (column 6) agree with those from ditype tetrads.

Examples 8–16 show, in contrast, how linkage may be overlooked if half tetrads rather than ditype tetrads are used as a criterion. (Linkage has in fact not previously been recognized in several of these cases, although ditype ratios indicate that it is highly probable.) The lesser significance of the half-tetrad deviations is due to including data from tetratype tetrads, which contribute parental and non-parental halves in equal numbers.

These applications support the contention that ditype tetrad numbers are generally more reliable and more efficient than half-tetrad numbers for indicating linkage. RIZET and ENGELMANN (1949, pp. 242, 257) have clearly recognized the role of ditype tetrads in linkage detection, and CATCHESIDE (1951, p. 25), following LINDEGREN (1933), has also noted that these two

tetrad classes provide a useful criterion of non-independence, but has unfortunately restricted his comparison to tetrads in which genes at both loci have segregated at the first meiotic division.

In practice, ditype numbers are extremely simple and convenient to use for determining the probability of linkage. Table 2 lists the lowest numerical ratios that deviate in one direction from 1 : 1 sufficiently to attain each of three confidence levels. These are the smallest PD : NPD ratios capable of providing

TABLE 2

Smallest numerical ratios showing significant deviation in one direction from 1 : 1.

Total numbers	Ratios attaining significance level (one-sided)			Total numbers	Ratios attaining significance level (one-sided)		
	5%	2 1/2%	1%		5%	2 1/2%	1%
5	5:0	....	....	28	19:9	20:8	21:7
6	6:0	6:0	....	29	20:9	21:8	22:7
7	7:0	7:0	7:0	30	20:10	21:9	22:8
8	7:1	8:0	8:0	31	21:10	22:9	23:8
9	8:1	8:1	9:0	32	22:10	22:10	23:9
10	9:1	9:1	10:0	33	22:11	23:10	24:9
11	9:2	10:1	10:1	34	23:11	24:10	25:9
12	10:2	10:2	11:1	35	23:12	24:11	25:10
13	10:3	11:2	12:1	36	24:12	25:11	26:10
14	11:3	12:2	12:2	37	24:13	25:12	26:11
15	12:3	12:3	13:2	38	25:13	26:12	27:11
16	12:4	13:3	14:2	39	26:13	27:12	28:11
17	13:4	13:4	14:3	40	26:14	27:13	28:12
18	13:5	14:4	15:3	41	27:14	28:13	29:12
19	14:5	15:4	15:4	42	27:15	28:14	29:13
20	15:5	15:5	16:4	43	28:15	29:14	30:13
21	15:6	16:5	17:4	44	28:16	29:15	31:13
22	16:6	17:5	17:5	45	29:16	30:15	31:14
23	16:7	17:6	18:5	46	30:16	31:15	32:14
24	17:7	18:6	19:5	47	30:17	31:16	32:15
25	18:7	18:7	19:6	48	31:17	32:16	33:15
26	18:8	19:7	20:6	49	31:18	32:17	34:15
27	19:8	20:7	20:7	50	32:18	33:17	34:16

significant indications of linkage. The values in table 2, obtained by using WARWICK's (1932) tables, can be determined with equal accuracy from binomial probability paper (MOSTELLER and TUKEY 1949), which is also useful for handling numbers beyond 50.

UTILITY OF RANDOM SINGLE STRANDS VERSUS TETRADS FOR ESTABLISHING LINKAGE

We have till now been concerned with the single problem, how best to detect linkage when segregants have been collected as tetrads. A second, distinct question can now be examined. Are tetrads preferable to random isolates for revealing linkage? Comparative efficiencies and reliabilities will be important for the choice, as will the prospect of obtaining other types of information from the same data used to establish linkage.

TABLE 3

Smallest numbers of tetrads and of random single strands likely to indicate linkage across intervals having specified exchange distributions.

Example no.*	Exchanges distributed among bivalents as for:	Length of interval**	Segregation probabilities	Smallest numbers likely to provide significant evidence of linkage***				Relative efficiency, randoms: tetrads
				Random strands		Ditype tetrads		
			PD : NPD : T	P + R	Total	PD + NPD	Total tetrads	
1	2	3	4	5		6		7	8
1	Alleles	0	1 : 0 : 0	5 + 0	5	5 + 0	5	5	4.0
2	Zero interference	25	0.628 : 0.021 : 0.352	7 + 1	8	5 + 0	5	8	4.0
3	" "	50	0.425 : 0.057 : 0.517	15 + 6	21	5 + 0	5	10	1.9
4	" "	100	0.251 : 0.116 : 0.633	89 + 67	156	16 + 7	23	63	1.6
5	" "	125	0.216 : 0.134 : 0.650	218 + 184	402	35 + 21	56	161	1.6
6	Complete interference	12.5	0.75 : 0 : 0.25	5 + 0	5	5 + 0	5	7	5.6
7	" "	25.0	0.50 : 0 : 0.50	11 + 3	14	5 + 0	5	10	2.9
8	" "	37.5	0.25 : 0 : 0.75	28 + 16	44	5 + 0	5	20	1.8
9	" "	45.0	0.10 : 0 : 0.90	154 + 125	279	5 + 0	5	50	0.7
10	Saccharomyces, *pn-in*	26	0.488 : 0.000 : 0.512	10 + 3	13	5 + 0	5	10	3.1
11	" , *g-me*	>46	0.230 : 0.147 : 0.623	219 + 185	404	44 + 28	72	192	1.9
12	Glomerella, Con.A-B	>45	0.211 : 0.106 : 0.683	143 + 115	258	23 + 11	34	108	1.7
13	Chlamydomonas, *b-mt*	>37	0.346 : 0.077 : 0.576	30 + 17	47	7 + 1	8	19	1.6
14	Schizophyllum, *A-s*	>27	0.500 : 0.045 : 0.455	12 + 4	16	5 + 0	5	9	2.2
15	Marchantia, *he-ac*	>27	0.474 : 0.013 : 0.513	10 + 3	13	5 + 0	5	10	3.1
16	Neurospora, *c-mt*	12	0.765 : 0.009 : 0.226	5 + 0	5	5 + 0	5	6	4.8
17	Zea, lg_1-v_4.	>60	0.182 : 0.054 : 0.764	97 + 74	171	9 + 2	11	47	1.1
18	Drosophila, *sc-f*	62	0.141 : 0.072 : 0.787	314 + 273	587	19 + 9	28	133	0.9
19	" , *y-bb*	>77	0.173 : 0.117 : 0.710	460 + 410	870	47 + 31	78	270	1.2
20	*Allium macranthum*, chiasmata, short chromosomes	70.4	0.140 : 0.109 : 0.750	1418 + 1330	2748	101 + 78	179	717	1.0
21	Chorthippus, chiasmata, short chromosome	52.0	0.010 : 0.010 : 0.980	....	....	....	....	....	....

*Sources of data: Ex. 10, 11, Lindegren 1949. Ex. 12, Chilton et al. 1949. Ex. 13, Lewin 1950. Ex. 14, Papazian 1951. Ex. 15, Burgeff 1943. Ex. 16, Mather et al. 1942. Ex. 17, Rhoades 1933. Ex. 18, Bridges et al. 1926. Ex. 19, Reck 1936. Ex. 20, Levan 1933. Ex. 21, Darlington 1932. Segregation probabilities in 17–19 are based on exchange frequencies calculated from genetic data by Ludwig (1938, p. 142).

**Values in map-units are from the experiments cited.

***$P \leq 0.05$, one-sided, for deviation from 1 : 1, and $P \geq 0.50$ for attaining or exceeding these proportions, on the basis of the ratios calculated from column 4. See appendix regarding computations.

Comparative efficiencies for linkage detection. Tetrads are less efficient than random strands for estimating linkage intensity (MATHER and BEALE 1942; PAPAZIAN 1952), but efficiencies of the two for *detecting* linkage have evidently never been compared. Table 3 gives the smallest numbers of tetrads and of random single strands likely to reveal linkage over various distances, for hypothetical models with complete interference (examples 6–9) and with none (2–5), and for cases where the frequencies of segregation types are known from genetic (10–19) or from cytological (20, 21) data. (Calculations for table 3 are described in an appendix.) These examples indicate that linkage can ordinarily be detected more efficiently with random single strands than with tetrads, i.e., that the amount of information per strand is greater for strands collected singly.

Three factors appear to be important in determining the relative efficiencies of tetrads compared to random isolates. (a) *The non-independence of constituent parts of tetrads.* About four times as many strands must usually be examined when tetrads are analyzed as when strands are collected at random, in order to reveal linkage over short distances (table 3, examples 1, 2, 6, 16). (b) *The interval length between markers.* Information per strand decreases from a maximum at short distances to zero for long intervals, both for random isolates and tetrads. The advantage of random strands over tetrads accordingly diminishes with distance (examples 2–5, 6–9, 10–11) until linkage can no longer be detected by either method across an interval where one or more exchanges occur in every bivalent (example 21). (c) *The intensity and pattern of interference.* Efficiencies of the two methods may be affected differentially by interference, so that tetrads can sometimes achieve or exceed the efficiency of random strands in the case of long intervals having a predominance of tetratype segregations (examples 9, 18).

In practice, the choice between tetrads and random strands will not depend solely on theoretical efficiencies (information per strand), but also on how laborious it is to collect strands by the two methods. In most organisms (liverworts are a possible exception) less work is required to obtain four strands at random than to isolate the four strands that compose a tetrad. The superior theoretical efficiency of random isolates relative to tetrads may thus be amplified by the greater ease with which random products can be obtained experimentally.

Sources of error in linkage detection. Differential viability or false identification could lead to serious errors either with tetrads or with single strands. Selection may operate either between individual segregants or between tetrads. While tetrad analysis sometimes makes it possible to identify a missing product by inference, and thus to decrease errors due to differential survival of particular segregant types, selection may also operate *among* tetrads against particular segregation classes that fail to produce complete complements of progeny. Such inter-tetrad selection might result, for example, from rejecting Neurospora asci having fewer than eight, or fewer than six, normal spores, and

could produce PD: NPD tetrad ratios that were more seriously in error than random-strand ratios from the same cross.

Whole tetrads seem less likely to be misclassified than single strands, but misidentification (or irregular segregation) could distort tetrad ratios if the constitution of incomplete tetrads is deduced by filling in missing products.

Other features of tetrads that may influence a choice of methods. Since random strands are usually more efficient than tetrads for linkage detection, and are perhaps no more subject to systematic errors, they would probably be preferred to tetrads if establishment of linkage were an isolated aim. But considerations other than accuracy and efficiency for detecting linkage may influence the choice.

Tetrads possess a number of advantages over random strands. Tetrad analysis is important for studying chromatid interference and for mapping centromeres (LINDEGREN 1933; WHITEHOUSE 1942; PAPAZIAN 1952). If a marker is available that regularly segregates with the centromere on one chromosome, the position of the centromere on any other chromosome can be mapped, even with unordered tetrads (e.g., KNAPP 1936). Efficiency is gained in mapping when the centromere distance of a gene is known so that tests can be made for linkage with specific markers that have similar centromere distances.

Tetrad analysis may indicate that particular variants result from crossing-over within a compound locus rather than from point mutation (PAPAZIAN 1951), even though the locus is not bridged with markers. (This is the simplest hypothesis where two reciprocally different new "alleles" appear as members of one tetrad.)

Tetrads show directly that crossing-over occurs between chromatids at the four-strand stage of meiosis, and segregation ratios in tetrads, being absolute rather than statistical, provide the most direct possible demonstration of the Mendelian basis of an inherited difference, as was recently pointed out by Quintanilha (cited in GUSTAFSSON 1951). Conversely, tetrads furnish direct evidence of extrachromosomal inheritance (CHEN et al. 1950). Aberrations or lethal mutations may be revealed that are undetectable with single strands (KNAPP 1937). Tetrads also make it possible to correct map-distance estimates for the occurrence of those double crossovers that do not result in recombination of the markers bounding an interval (PERKINS 1949).

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SUMMARY

When complete tetrads are analyzed, the most satisfactory criterion of departures from random segregation is a significant deviation from equality

of parental and non-parental ditype tetrads. Treatment of tetrad data as though the component half tetrads or single strands were independent is not valid for purposes of linkage detection, and may either obscure the existence of linkage, or indicate linkage spuriously where none obtains.

Linkage can ordinarily be detected more efficiently with random single strands than with tetrads. Nevertheless, tetrads may sometimes be preferred because of their superiority for purposes other than linkage detection.

APPENDIX: CALCULATIONS FOR TABLE 3

Distribution of exchanges among bivalents. Relative proportions of PD, NPD and T segregations (column 4) are obtained directly only when tetrads are analyzed (examples 10–16), but can be computed if the proportions are known of bivalents having different numbers of exchanges, i.e., bivalents of different rank (WEINSTEIN 1936). Relative frequencies of different ranks can be estimated from chiasma counts (examples 20, 21), or from multiple-point single-strand crossover data (MATHER 1933; WEINSTEIN 1936; LUDWIG 1938; examples 17–19). They are also known for complete interference (examples 6–9) where all exchanges occur as singles, and for zero interference (examples 2–5) where exchanges would be distributed among the bivalents at random, and probabilities for occurrence of bivalents of increasing rank are given by the successive terms of a Poisson series.

Calculation of tetrad proportions if the distribution of exchange frequencies is known. The probability t_r that a bivalent of rank r will segregate as a tetratype can be obtained from chromosome diagrams, or by use of the progression $t_r = (2/3)\ [1 - (-1/2)^r]$ adapted from Mather (1935). For $r > 0$, each ditype has a probability of occurrence $d_r = (1 - t_r)/2$, but for linked genes, all segregations are parental ditypes when $r = 0$. If one knows the probabilities of occurrence of bivalents of different rank, P_r, these equations give the corresponding probabilities of obtaining each of the 3 segregation types—PD, NPD or T—so that appropriate probabilities for individual ranks can be multiplied, and contributions from bivalents of all ranks then summed, to obtain overall probabilities for occurrence of each tetrad type in a specified cross. The probability of tetratypes, $t = \sum_{r=0}^{\infty} P_r t_r$, the probability of NPD tetrads $= d = \sum_{r=1}^{\infty} P_r d_r$, and the probability of PD tetrads $= d + P_0$. Column 4 values in examples 2–9 and 17–21 were obtained in this way.

Smallest numbers of random strands likely to indicate linkage (Column 5). The probabilities of obtaining parental strands from PD, NPD and T segregations are 1, 0 and 0.5 respectively, whence ratios of parental : recombinant strands can be calculated from tetrad ratios. It is desired to obtain, for column 5, the smallest number of random isolates expected to indicate linkage in at least 50% of tests. The smallest R : P numbers likely ($P \geq 0.5$ for attainment if segregations are as in column 4) to show a significant deviation from 1 : 1 ($P \leq 0.05$, one-sided) can readily be determined graphically, using MOSTELLER and TUKEY'S (1949) binomial probability paper, and determining the paired count whose apex falls between and beyond the intersection of the R : P split and a line paralleling the 50 : 50 split at 8.4 mm distance.

Smallest numbers of ditype tetrads likely to indicate linkage (Column 6). NPD : PD ratios from column 4 are used directly to plot a split on binomial probability paper, and the smallest paired count is determined as described above.

Smallest total number of tetrads required (Column 7). Once minimum ditype numbers have been specified, the total number of tetrads that must be analyzed to provide enough ditypes depends in turn upon the tetratype proportions. Again, binomial probability paper can be used, plotting the tetratype : ditype split and determining the paired

count nearest the point where this split intersects a horizonal line passing through the minimum ditype number.

Relative efficiencies (Column 8). Since information per strand is inversely proportional to the number of strands required, the relative efficiency of *random strands : tetrads* is expressed as the proportion: (*total strands required as tetrads*)/(*total strands required as randoms*). Graphical and arithmetic errors are small compared to the differences in efficiency.

The unexpected appearance of relative efficiencies exceeding four (examples 6, 16) is due to the fact that calculations are based on the smallest numbers of random strands and of tetrads that are likely ($P \geq 0.5$) to provide significant evidence of linkage. Relative efficiencies would attain but not exceed four if the comparison had been based on the *mean* numbers required to reveal linkage, rather than on the smallest numbers *likely* to do so. In general, results from the two measures of efficiency would differ but little. Conclusions drawn from table 3 would be the same whichever criterion was used.

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